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(54) Title: THE PREVENTION AND TREATMENT FOR RETINAL ISCHEMIA AND EDEMA

(57) Abstract

    The present invention relates to methods of treating retinopathy, retinal ischemia and/or retinal edema comprising administering an integrin or integrin subunit antagonist, leukocyte adhesion–inducing cytokine antagonist or growth factor antagonist, a selectin antagonist or adhesion molecule antagonist.

THE PREVENTION AND TREATMENT FOR RETINAL ISCHEMIA

AND EDEMA

RELATED APPLICATION

This application is a Continuation-in-Part of U.S. Application No.
5   09/248,752, filed February 12, 1999, entitled, "The Prevention and Treatment for
Retinal Ischemia and Edema," and claims the benefit of U.S. Provisional
Application No. 60/114,221, filed December 30, 1998, entitled, "The Prevention and
Treatment for Retinal Ischemia and Edema," the entire teachings of which are
incorporated herein by reference.

10   BACKGROUND OF THE INVENTION

Diabetes affects over 16 million Americans. The World Health Organization
indicates that diabetes afflicts 120 million people worldwide, and estimates that this
number will increase to 300 million by the year 2025. Diabetics are faced with
numerous complications including kidney failure, non-traumatic amputations, an
15   increase in the incidence of heart attack or stroke, nerve damage, and loss of vision.
Diabetic retinopathy is a form of visual impairment suffered by diabetics.

In particular, diabetic retinopathy is responsible for 13.1% and 18.2% newly
reported cases of blindness for men and women, respectively. Kohner E.M., et al.
Diabetic Retinopathy Metabolism, 25:1985-1102 (1975). The prevalence of blind
20   diabetics in the population is about 100 people per million. Id.

Less than optimal methods of treatment for diabetic retinopathy exist. For
example, laser treatment may be used to slow the progression of edema, but it
cannot be used to reverse the symptoms of diabetes. Accordingly, a need exists to
develop effective methods of treatment to reduce or impede vision loss and/or
25   diabetic retinopathy.

–2–

SUMMARY OF THE INVENTION

The present invention relates to methods for inhibiting the binding of a leukocyte to an endothelial cell or another leukocyte in the retinal vasculature. The present invention pertains to methods of treating (e.g., reducing or preventing)

5 retinal injury in a mammal (e.g., human, individual, patient) wherein the injury involves retinal edema or retinal ischemia, comprising administering a compound that inhibits the binding of a leukocyte to endothelium or to another leukocyte wherein a reduction in edema or ischemia (e.g., non-perfusion) occurs. The compound comprises an integrin antagonist (e.g., lymphocyte function associated

10 molecule-1 (LFA-1), Mac-1 or p150,95), a selectin (e.g., P-selectin, E-selectin and L-selectin) antagonist, an adhesion molecule antagonist (e.g., Intercellular Adhesion Molecule (ICAM)-1, ICAM-2, ICAM-3, Platelet Endothelial Adhesion Molecule (PCAM), Vascular Cell Adhesion Molecule (VCAM)), or a leukocyte adhesion-inducing cytokine or growth factor antagonist (e.g., Tumor Neucrosis

15 Factor-α (TNF-α), Interleukin-1 β (IL-1 β), Monocyte Chemotatic Protein-1 (MCP-1) and a Vascular Endothelial Growth Factor (VEGF)). The integrin antagonist can be an integrin subunit (e.g., CD18 or a CD11b) antagonist. The antagonist can be administered with or without a carrier (e.g., pharmaceutically acceptable carrier).

In particular, the invention pertains to methods of treating or preventing

20 retinal injury in a mammal comprising administering to the mammal an adhesion molecule antagonist and/or an integrin antagonist, wherein the adhesion molecule antagonist and/or the integrin antagonist inhibits leukocyte interaction, thereby reducing or preventing retinal injury. The antagonist can be administered in a carrier (e.g., a pharmaceutically acceptable carrier). The antagonist for adhesion

25 molecule can be a VCAM, PCAM, ICAM-2 or ICAM-3 antagonist or, preferably, an ICAM-1 antagonist. In particular, the antagonist can be an antibody or an antibody fragment which is specific for ICAM-1, an antisense molecule that hybridizes to the nucleic acid sequence which encodes ICAM-1, or a peptide mimetics molecule, a ribozyme, an aptamer, or a small molecule antagonist that inhibits ICAM-1. The

30 integrin antagonist can be a LFA-1 antagonist , Mac-1 antagonist or p150,95 antagonist. The integrin antagonist also comprises an integrin subunit antagonist

–3–

(e.g.,a CD18 antagonist and/or a CD11b antagonist). The antagonist can be an
antibody or antibody fragment specific for CD18 and/or CD11b, an antisense
molecule that hybridizes to the nucleic acid sequence that encodes CD18 and/or
CD11b, or a peptide mimetic molecule, a ribozyme, an aptamer or a small molecule
5 antagonist that inhibits CD18 or CD11b.

Another aspect of the invention includes a method for preventing or treating
an individual having retinal injury (e.g., injury caused by diabetic retinopathy),
wherein the injury is associated with retinal edema and/or retinal ischemia,
comprising administering to the individual a compound that inhibits Mac-1 or a
10 pathway thereof. The compound inhibits ICAM-1, CD18, CD11b, and/or VEGF,
and causes a decrease of ischemia and/or edema (e.g., between about 10% and about
90%). Leukocyte interaction can also be reduced. The compound can be an
antibody, an antibody fragment, a peptide mimetic molecule, an antisense molecule,
a ribozyme, an aptamer and/or a small molecule antagonist. Examples for such a
15 compound are ICAM-1, CD18, CD11b, and/or VEGF.

The invention also pertains to a method of treating an individual having
retinopathy or at risk for retinopathy (e.g., diabetic retinopathy) comprising
administering an antagonist (e.g., ICAM-1, CD18, CD11b and/or VEGF), as
described herein. The antagonist can optionally be administered in a suitable carrier
20 (e.g., pharmaceutically acceptable carrier). Administration of this antagonist results
in a decrease in retinal ischemia and/or retinal edema. Preferably, a decrease in
ischemia and/or edema occurs by at least about 10%, and more preferably, by about
20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% (e.g., between 10% and 95%).
Accordingly, the present invention also relates to methods for treating or preventing
25 retinal edema and/or retinal ischemia comprising administering an ICAM antagonist
(e.g., ICAM-1), a CD18 antagonist, a CD11b antagonist and/or a VEGF antagonist,
wherein a decrease in the edema and/or ischemia occurs.

The present invention also relates to methods of treating diabetic retinopathy
by administering an ICAM-1, CD18, CD11b and/or VEGF antagonist and at least
30 one additional antagonist that inhibits the binding of a leukocyte to an endothelial
cell or to another leukocyte. The additional antagonist can be an integrin antagonist

(e.g., an integrin subunit antagonist such as CD18 and/or CD11b), a selectin
antagonist, a leukocyte adhesion-inducing or growth factor antagonist, or adhesion
molecule antagonist. The additional antagonist can be, for example, another ICAM
antagonist (e.g., an antagonist that is specific for a different portion or epitope of the
5   ICAM-1 molecule), a PCAM antagonist or a VCAM antagonist. The types of
integrin antagonists, selectin antagonists, and leukocyte adhesion-inducing or
growth factor antagonists are described herein.

         The invention also encompasses a method of inhibiting leukocyte interaction,
comprising contacting a leukocyte, an endothelial cell or a leukocyte
10   adhesion-inducing cytokine, with a compound or antagonist, as defined herein. The
compound can be an integrin antagonist (e.g., an integrin sub-unit antagonist such
as CD18 and/or CD11b), a selectin antagonist, an adhesion molecule antagonist or a
leukocyte adhesion-inducing cytokine or growth factor antagonist. In particular, the
invention relates to a method of inhibiting leukocyte interaction, comprising
15   contacting an endothelial cell with an adhesion molecule antagonist (e.g., ICAM-1
specific antagonist), an integrin subunit antagonist (e.g., CD18 and/or CD11b
specific antagonist), or a leukocyte adhesion-inducing cytokine antagonist or growth
factor antagonist (e.g., TNF-1α , IL-1β, MCP-1 and VEGF antagonist).

         The invention also pertains to a method of preventing or reducing retinal
20   leukostasis an a mammal comprising administering to the mammal an effective
amount of an ICAM, CD18, CD11b and/or VEGF antagonist. The types of
antagonist is described herein. The method results in retinal leukostasis reduction
by between about 10% and 90%.

         Another aspect of the invention is a method of decreasing retinal leukocyte
25   adhesion in a mammal, comprising administering to the mammal an effective
amount of an antagonist that is specific for CD11b, CD18 or a combination thereof.
The retinal leukocyte adhesion is decreased between about 10% and 90%.

         Yet another aspect of the invention is a method of treating or preventing
neovascularization in a mammal, comprising administering to the mammal a CD18
30   antagonist and an ICAM-1 antagonist, or a CD18 antagonist. The types of
antagonists are described herein. The method is applicable to diseases or conditions

associated with neovascularization including, but not limited to, age-related macular degeneration, choroidal neovascularization, sickle cell retinopathy, retina vein occlusion, diabetic retinopathy, a condition associated with limbal injury, a condition associated with increased neovascularization, traumatic alkali injury,

5   Stevens Johnson syndrome and ocular cicatricial pemphagoid. The neovascularization can be reduced in the cornea, the retina or the choroid.

Advantages of the present invention include effective treatment for retinopathy, retinal edema, retinal ischemia, neovascularization and other associated disease. Treatment of these diseases and/or conditions have been ineffective until the

10   discovery of the present invention. For the first time, the present invention provides useful methods of treatment which target molecules that are involved in these diseases.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a graph showing the density of trapped leukocytes as measured

15   on 0, 3, 7, 14, 21 and 28 days after diabetes induction. The graph shows a time course of diabetic retinal leukostasis. All data show the mean ± the standard deviation (SD).

Figure 1B is a graph showing the retinal vascular [^{125}I] albumin permeation measured 0, 3, 7, 14, 21 and 28 days after diabetes induction. The graph shows a

20   time course of vascular leakage. All data show the mean ± the standard deviation (SD).

Figures 2A-D show four photographs of the same retinal area. Figure 2A and Figure 2B show the retinal area after seven days of diabetes. Figure 2C and Figure 2D show the retinal area after eight days of diabetes. Figures 2A and Figures

25   2C are photographs from orange leukocyte fluorography (AOLF) and Figures 2B and Figures 2D are photographs from fluorescein angiography. Scale bar denotes 100 μm (3.2 pixel = 1μm).

Figure 3A-F show six photographs of a retinal area. Figures 3A and 3B show the retinal area after one week, Figures 3C and 3D show the retinal area after

30   two weeks, and Figures 3E and 3F show the retinal area after four weeks. Figures

−6−

3A, 3C, and 3E are photographs from AOLF and Figures 3B, 3D and 3F are
photographs from fluorescein angiography. Scale bar denotes 100 μm (3.2 pixel = 1
μm).

Figure 4A is a photograph of ribonuclease protection assay results showing
ICAM-1 mRNA levels from controls and a diabetic rat three days following diabetes
induction. Each lane is the signal from the two retinas of a single animal. The lane
labeled "Probes" shows a hundred-fold dilution of the full-length ICAM-1 and 18S
riboprobes. The lanes labeled "RNase-(0.1)" and "RNase-(0.01)" show the ten-fold
and hundred-fold dilutions, respectively, of the full length riboprobes without
sample or RNase.

Figure 4B is a bar graph showing units of normalized ICAM-1 mRNA for
controls, three days and seven days after diabetes induction.

Figures 5A-D are photographs of the retinal area. The representative retinal
leukostasis is shown in non-diabetic test subjects (Figure 5A), diabetic test subjects
(Figure 5B), diabetic test subjects given 5 mg/kg mouse control IgG1 (Figure 5C)
and diabetic test subjects treated with 5 mg/kg anti-ICAM-1 mAb-treated animals
(Figure 5D). Scale Bars = 100 μm; 3.2 pixel = 1 μm.

Figure 6A is a bar graph showing the density of trapped leukocytes ($x10^{-5}$
cells/pixel^2) for control, diabetic test subjects not given anything, diabetic test
subjects given 5mg/kg mouse IgG1, diabetic test subjects treated with 3 mg/kg anti-
ICAM-1 antibody, and diabetic test subjects treated with 5 mg/kg anti-ICAM-1
antibody. NS= Not Significant.

Figure 6B is a bar graph showing the retinal vascular ^{125}I albumin permeation
(μg plasma xg tissue wet weight^{-1} x min^{-1}) for control, diabetic test subjects not
given anything, diabetic test subjects given 5mg/kg mouse IgG1, diabetic test
subjects treated with 3 mg/kg anti-ICAM-1 antibody, and diabetic test subjects
treated with 5 mg/kg anti-ICAM-1 antibody. NS= Not Significant.

Figure 7 is a bar graph showing the amount of adherent neutrophils to
endothelium *in vitro* (thousands per mm^2) from control rats and rats having Diabetes
Mellitus (DM). All data shown are means ±Standard Deviation (SD).

Figure 8 is a bar graph showing the amount of adherent neutrophils

−7−

(thousands per mm^2) for untreated, CD11a, CD11b, CD18, or CD11a/CD11b/CD18 cocktail treated for control and DM rats. All data shown are means ± SD.

Figures 9A-D are photographs of leukostasis in from AOLF retinas in non-diabetic rat (Figure 9A), diabetic rat (Figure 9B), diabetic rat treated with the control F(ab')$_2$ (Figure 9C) and anti-CD18 F(ab')$_2$ fragments treated rats (Figure 9D).

Figure 10 is a bar graph showing the density fo trapped leukocytes (x10^{-5} cells/pixel^2) for control, DM, DM and F(ab')$_2$, and DM and anti-CD18 F(ab')$_2$ fragment treated rats.

Figures 11A-B are photographs of AOLF retina before (Figure 11A) and 48 hours after a 50 ng Vascular Endothelial Growth Factor (VEGF) injection (Figure 11B). Scale bar denotes 100 μm (3.2 pixel=1 μm).

Figure 12 is a bar graph showing the density of trapped leukocytes (x10^{-5} cells/pixel^2, mean =SD) in the retina using AOLF for rats injected intravitreously with 0, 5, 10, 50, 100 ng of VEGF after 48 hours.

Figure 13 is a bar graph showing the density of trapped leukocytes (x10^{-5} cells/pixel^2, mean =SD) in the retina using AOLF for rats injected intravitreously with the vehicle alone or with 50 ng of VEGF after 6, 24, 48, 72, or 120 hours.

Figure 14 is a bar graph showing the density of trapped leukocytes (x10^{-5} cells/pixel^2, mean =SD) in the retina using AOLF for rats injected intravitreously with the vehicle alone or 50 ng of VEGF with and without anti-VEGF mAb treatment after 48 hours.

Figures 15A-B are photographs of retina 48 hours after rats were injected intravitreously with 50 ng (Figure 15A) followed by fluorescein angiography (Figure 15B). Arrows indicate areas of capillary non-perfusion downstream from static leukocytes. Scale bar denotes 100 μm (3.2 pixel=1 μm).

Figures 16A-B show VEGF-induced retinal ICAM-1 gen expression. Figure 16A is a photograph showing results of a ribonuclease protection that demonstrated that retinal ICAM-1 levels were significantly increased 20 h following the intravitreous delivery of 50 ng VEGF. Control animals received 5 μl of PBS solvent alone. Each lane shows the signal from one retina of one animal. The lane labeled "Probes" shows a hundred-fold dilution of the full-length ICAM-1 and 18S

–8–

riboprobes. The lanes labeled "RNase - (0.1)" and "RNase - (0.01)" show the ten-
fold and hundred-fold dilutions, respectively, of the full-length riboprobes without
sample or RNase. The lane labeled "RNase +" shows the full-length riboprobes with
RNase, but without sample. Figure 16B is a bar graph showing the amount of
5   normalized ICAM-1 mRNA in the retina (arbitrary units, mean + SD) for rats
injected with the vehicle alone and with 50 ng of VEGF. NS = not significant.

Figures 17A-B are bar graphs showing the effect of anti-ICAM-1 mAb on
permeability and leukostasis following intravitreous VEGF injection. Figure 17A is
a bar graph showing the retinal vascular [^{125}I] albumin permeation (μg plasma x g
10   tissue wet weight $^{-1}$ x min^{-1}, mean + SD) for rats that were untreated, or treated with
the vehicle alone, 50 ng VEGF, 50 VEGF and mouse IgG1, or 50ng VEGF and an
anti-ICAM-1 antibody. Figure 17B is a bar graph showing the density of trapped
leukocytes (x10^{-5} cells/pixel^2, mean =SD) in the retina using AOLF for untreated rats
or rats treated with treated with the vehicle alone, 50 ng VEGF, 50 VEGF and
15   mouse IgG1, or 50ng VEGF and an anti-ICAM-1 antibody. ICAM-1 bioactivity
was inhibited via intravenous administration of ICAM-1 neutralizing antibody and
retinal permeability (Figure 17A) or leukostasis (Figure 17B) were evaluated,
respectively. NS = not significant.

DETAILED DESCRIPTION OF THE INVENTION
20   The invention relates to methods of treating and/or preventing retinal injury
in a mammal by administering to the mammal a compound that inhibits leukocyte
interaction which is the binding of a leukocyte to an endothelial cell or to another
leukocyte. Several antagonists inhibit leukocyte interaction and include an integrin
antagonist, a selectin antagonist, an adhesion molecule antagonist, or a leukocyte
25   adhesion-inducing cytokine antagonist or growth factor antagonist. In particular, an
intercellular adhesion molecule-1 antagonist, a CD18 antagonist, a CD11b
antagonist or a VEGF antagonist are encompassed by the present method.
Administration of such antagonists results in a significant decrease in retinal edema
and/or retinal ischemia. The retinal injury can be caused by retinopathy or a
30   visually-related disease that involves leukocyte occlusion in blood vessels (e.g.,

–9–

capillaries) and their destruction (e.g., atrophy). In particular, the present methods
pertain to treating diabetic retinopathy.

     Diabetic retinopathy is a progressive degeneration of retinal blood vessels
and is a consequence of diabetes, in particular, diabetes mellitus. One important

5   aspect of the disease is retinal edema. Fluid build up from deteriorating blood
vessels and capillaries causes edema. As the disease progresses, the damage
proliferates and large hemorrhages and retinal detachment can result.

     The term "retinopathy" also refers to noninflammatory degenerative diseases
of the retina. The methods of the present invention encompass retinopathy or a

10   visually-related disease that is characterized by one or more of the following retinal
signs: capillary obstruction, nonperfusion, leukostasis, formation of vascular lesions
and/or proliferation of new blood vessels in association with ischemic areas of the
retina. Leukostasis refers to the stasis or non-movement of white blood cells (e.g.,
leukocytes) in the vasculature. Other disorders or diseases implicated by the

15   invention involve diseases which result in retinal edema and/or retinal ischemia.
Examples of such diseases include vein occlusions, sickle cell retinopathy, radiation
retinopathy, diabetic retinopathy, VEGF-induced diseases and retinopathy
prematurity.

     Capillary occlusions constitute a characteristic pathologic feature in diabetic

20   retinopathy, and, when widespread, initiate neovascularization. Neovascularization
(e.g., angiogenesis) refers to the formation or growth of new blood vessels.
Microaneurysms, intraretinal microvascular abnormalities and vasodilation also are
commonly found in early stages of diabetic retinopathy and have been correlated to
capillary occlusions. Schroder, S. *et al.*, *American Journal of Pathology*, 139 (81),

25   81-100 (1991). Leukocytes cause capillary obstruction that is involved in diabetic
retinopathy via two mechanisms. This obstruction is the result of the leukocytes'
large cells volume and high cytoplasmic rigidity. Leukocytes can become trapped in
capillaries under conditions of reduced perfusion pressure (e.g., caused by
vasoconstriction) or in the presence of elevated adhesive stress between leukocytes

30   and the endothelium, endothelial swelling, or narrowing of the capillary lumen by
perivascular edema. Id. Examples of leukocytes include granulocytes,

−10−

lymphocytes, monocytes, neutrophils, eosinophils, and basophils. Elevated adhesive stress can result from release of chemotactic factors or expression of adhesion molecules on leukocytes or endothelial cells. Secondly, leukocytes injures capillaries leading to capillary death, also known as "capillary dropout."

5   A number of glycoproteins are involved in the adhesion of leukocytes. In the case of neutrophils and monocytes, a family of glycoproteins, known as β_2 integrins, have been identified. This family of integrins include Lymphocyte Function Associated Antigen-1 (LFA-1), Mac-1, and p150,95. Some integrins are made up of molecules referred to as "subunits" or "integrin subunits." The LFA-1 integrin is
10   comprised of 2 subunits, CD11a and CD18, Mac-1 integrin is comprised of CD11b and CD18, and p150,95 is made up of CD11c and CD18.

        A corresponding family of glycoproteins, referred to as selectins, are expressed in endothelial cells or can be induced by stimulation with endotoxins or cytokines. The selectins include P-selectin, E-selectin, and L-selectin. The selectin
15   family is involved in endothelial interaction. Firm adhesion of activated polymorphonuclear neutrophils (PMN) to the endothelial cells occur through the interaction between integrins (e.g., LFA-1, MAC-1 and p150,95) expressed on the PMNs and members of the immunoglobulin superfamily of proteins, referred to as Intercellular Adhesion Molecule-1 (ICAM-1), Platelet Endothelial Adhesion
20   Molecule (PCAM), and Vascular Cell Adhesion Molecule (VCAM), expressed by the endothelium. Additionally, cytokines such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1 β (IL-1 β), Monocyte Chemotactic Protein-1 (MCP-1), and growth factors (VEGF) can induce the surface expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells.

25   Intercellular adhesion molecules are involved in and are important for inflammation responses. Mediators of inflammation cause an induction of ICAM-1 expression on various cell types and sites of inflammation. Both soluble and membrane forms of ICAM-1 exist. Roep, B.O. *et al.*, *Lancet* 343, 1590-1593, 1590 (1994). ICAM-1 is an inducible cell surface ligand for LFA-1. Larson, R.S., *et al.*,
30   *Immunological Reviews*, 114, 181-217, 192 (1990). ICAM-1 is a single chain glycoprotein with a peptide backbone of 55 kD. ICAM-1 is a member of

–11–

immunoglobulin super family consisting of 5 immunoglobulin-like domains. ICAM-1 is expressed or induced by inflammatory mediators on many cell types including endothelial cells, epithelial cells, keratinocytes, synovial cells, lymphocytes, and monocytes. The LFA-1 binding site is the first immunoglobulin
5   domain of ICAM-1. ICAM-1 also binds with Mac-1, an important mechanism in retinal edema and retinal ischemia. Various forms of ICAM-1 can be used to generate antagonists, such as antibodies or antisense molecules.

        Retinal leukostasis is a very early event in diabetic retinopathy with important functional consequences. Both retinal vascular leakage and non-perfusion
10 follow its development. The inhibition of ICAM-1 activity blocks diabetic retinal leukostasis and potently prevents blood-retinal barrier breakdown. Leukostasis is associated with the development of vascular nonperfusion and thus its inhibition can also prevent capillary dropout. Indeed, activated leukocytes are increased in diabetes and leukocytes have been associated with capillary loss in the diabetic choroid. The
15 data described herein demonstrate that ICAM-1-mediated leukostasis is increased in the retinal vasculature very early in diabetes and accounts for the majority of diabetes-associated retinal vascular leakage. Thus, these data, described herein, indicate ICAM-1 as a new therapeutic target for the prevention of many of the sight-threatening retinal abnormalities, especially those associated with diabetes. See
20 Example 1.

        The data described herein also show that CD11a, CD11b, and CD18 β_2 integrin levels were increased on the surface of neutrophils from diabetic rats. The increases correlated with the enhanced functional adhesiveness of diabetic neutrophils to rat endothelial cell monolayers. Similarly, in an *in vivo* model of
25 experimentally-induced diabetes, use of anti-CD18 F(ab')$_2$ fragments significantly decreased diabetic retinal leukostasis by 62%, confirming the relevance of the *in vitro* findings. The data described herein indicate that the Mac-1 integrin complex is operative in the adhesion of diabetic neutrophils to the retinal capillary endothelium. Since a major ligand for Mac-1 is ICAM-1, these results are consistent with data,
30 shown in Example 1, that ICAM-1 blockade prevents diabetic retinal leukostasis and blood-retinal barrier breakdown. See also Example 2.

–12–

Based on the data described herein, it is reasonable to believe that the leukocyte adhesive changes in this model of diabetes are of a systemic nature. The assayed neutrophils were isolated from the peripheral blood, and therefore reflected systemic neutrophil adhesion molecule expression and bioactivity. The causes of the

5   surface integrin changes remain unknown, however they are likely to be linked to hyperglycemia. For example, hyperglycemia directly impacts TNFα expression, a cytokine known to activate integrin adhesion molecules on leukocytes. *In vitro* work has also shown that hyperglycemia promotes increased leukocyte adhesion to endothelium via ICAM-1 and CD18. Thus, hyperglycemia, either directly or

10   indirectly, is a proximal stimulus for the ICAM-1 and CD18 upregulation seen in diabetes.

Also, these data show that a low-level retinal leukostasis occurs in the normal state. The same molecules that are operative in the diabetic state also mediate this presumably normal phenomenon. If the low-level leukostasis in the

15   non-diabetic state is physiologic, then the specificity of an anti-integrin therapy can be compromised.

The results, described herein, also provide additional evidence of leukocyte involvement in the pathogenesis of diabetic retinopathy. The aggregate data indicate that diabetic retinopathy should be, in one sense, redefined as an inflammatory

20   disease. Very early in diabetes leukocytes adhere to the vascular endothelium, trigger breakdown of the blood-retinal barrier, impede flow, and in some instances, extravasate into the retinal parenchyma. The identification of Mac-1 as a functional adhesive molecule in diabetic retinopathy provides a target for the prevention and/or treatment of the disease.

25   Data described herein also show that VEGF induces retinal vascular permeability and leukostasis through ICAM-1. Retinal leukostasis was also spatially linked to capillary non-perfusion. The vitreous concentration at which VEGF begins to induce these changes (12.5 nM) is within the range of vitreous VEGF concentrations observed in human eyes with diabetic retinopathy. The leukostasis

30   observed in these studies was specific to VEGF because co-injection of a neutralizing antibody abrogated the response. Finally, these findings are consistent

with our data showing VEGF-induced ICAM-1 expression in the retinal vasculature. See Example 3.

Leukocytes, via their own VEGF, serve to amplify the direct effects of VEGF when they bind to endothelium. VEGF has been demonstrated in neutrophils,

5   monocytes, eosinophils, lymphocytes and platelets. The fact that some leukocytes possess high affinity VEGF receptors and migrate in response to VEGF makes this scenario even more likely.

The data also show that VEGF-induced capillary non-perfusion occurs downstream from areas of leukocyte adhesion. Leukocyte-mediated non-perfusion

10 characterizes experimental diabetic retinopathy. In diabetes, patent capillaries become occluded downstream from newly arrived static leukocytes. Later, following the disappearance of the leukocytes, the capillaries reopen. Since neutrophil and monocyte diameters can exceed those of retinal capillary lumens, leukocyte-mediated flow impedance is a likely mechanism.

15 Taken together, these data indicate that VEGF-induced vascular permeability is mediated by ICAM-1-mediated retinal leukostasis. These data are the first to show that a non-endothelial cell type contributes to VEGF-induced vascular permeability. They are also the first to provide a mechanism for the capillary non-perfusion induced by VEGF. Given these findings, targeting ICAM-1 proves useful in the

20 treatment of diseases characterized by VEGF-induced vascular changes, such as diabetic retinopathy.

The invention takes advantage of the surprising discovery that inhibiting integrins, and in particular the Mac-1 or a pathway thereof, results in a reduction in retinal edema and/or retinal ischemia. This reduction in both retinal edema and/or

25 retinal ischemia provides an effective treatment for various ocular diseases, including retinopathy. In one aspect of the invention, an antagonist's biological activity refers to a compound that inhibits the Mac-1 integrin adhesion or a pathway thereof. Inhibition can occur directly (e.g., by inhibiting binding of the Mac-1 molecule or a subunit thereof such as CD18 or CD11b), or indirectly (e.g., by

30 inhibiting a molecule that affects Mac-1 such as by inhibiting ICAM-1 expression or Vascular Endothelial Growth Factor (VEGF) expression). The surprising results of

—14—

directly inhibiting Mac-1 by inhibiting Mac-1 subunits are shown in Example 2.
Several molecules indirectly impact on Mac-1's biological activity (e.g., its ability to
bind to ICAM-1, induce leukocyte adhesion, induce leukostasis, cause edema and/or
cause ischemia). For example, ICAM-1 directly binds to Mac-1. Inhibiting ICAM-
5   1 reduces retinal edema and ischemia. See Example 1. Similarly, VEGF mediates
ICAM-1 expression in the retinal vasculature, and induces vascular permeability and
non-perfusion. Inhibiting VEGF results in decreased expression of ICAM-1, and a
reduction in both retinal edema and retinal ischemia. See Example 3. Additionally,
TNF-α, a cytokine, induces ICAM-1 expression, which, in turn, can stimulate and
10   increase leukocyte adhesion. Inhibiting the TNF-α pathway, significantly reduces
leukocyte adhesion. See Example 2. Inhibiting Mac-1 and molecules that affect the
Mac-1 pathway (e.g., ICAM-1 expression) unexpectedly results in reductions of
retinal edema and ischemia.

        Inhibition of a molecule encompassed by the invention (e.g., Mac-1, CD18,
15   CD11b, ICAM-1, VEGF or TNF-α) can be accomplished in several ways. A
molecule can be made inactive or its action disrupted. For example, the expression
of these molecules can be inhibited prior to the molecule exiting the cell using, for
example, antisense technology, etc. The molecule can also be made inactive by
inhibiting its binding to a receptor after it exits the cell or is exposed on the
20   membrane of the cell, e.g., with an antibody or antibody fragment. Additionally, the
action of these molecules can be inhibited by disrupting the signaling downstream
from the receptor (e.g. alterations in phosphorylation). These and other methods can
be used so long as the activity or action of one or more of the molecule described
herein is inhibited or disrupted.

25   The invention relates to preventing or treating retinal injury wherein the
retinal injury involves retinal edema and/or retinal ischemia, comprising
administering a compound that inhibits the binding of a leukocyte to an endothelial
cell or another leukocyte in, for example, a blood vessel or capillary, which results
in the reduction of retinal edema and/or retinal ischemia. The term "retinal injury"
30   is defined herein as a decreased ability for the retina to function normally as
measured, for example, by the patient's vision, electrical signal potential, fluorescein

−15−

angiograms or other known methods or methods developed in the future. The
compound has the ability to inhibit or reduce leukocyte occlusion in the retinal
vasculature. As described herein, the compound can be an integrin antagonist (e.g.,
Mac-1 antagonist), an integrin subunit antagonist (e.g., CD18 antagonist or a CD11b

5   antagonist), a selectin antagonist, a leukocyte adhesion-inducing cytokine antagonist
or growth factor antagonist (e.g., TNF-α , IL-1β, MCP-1 and VEGF antagonist), or
an adhesion molecule antagonist (e.g., an ICAM-1, ICAM-2, ICAM-3, PCAM or
VCAM antagonist). In particular, the invention also pertains to administering an
ICAM-1 antagonist, a VEGF antagonist, a Mac-1 antagonist, a CD18 antagonist, or

10 a CD11b antagonist, to treat retinal edema, retinal ischemia, and/or diabetic
retinopathy. The various forms of the antagonists are described herein.

        The methods described herein can be used for treating ocular tissue that
experiences leukostasis, edema and/or ischemia. Such tissue includes the retina, and
the choroid. For example, the invention includes a method of treating or reducing

15 leukostasis, edema and/or ischemia in the retina or the choroid of an affected
mammal by administering to the mammal one or a combination of any one of the
antagonists described herein.

        The invention includes methods of inhibiting leukocyte interaction,
comprising contacting a leukocyte or endothelial cell with an antagonist. For

20 example, using the various antagonists described herein, one can contact a leukocyte
with an integrin antagonist, an endothelial cell with an adhesion molecule antagonist
or a selectin antagonist, or subject the cytokines that induce surface expression of
ICAM-1, VCAM-1, and E-selectin to a leukocyte adhesion-inducing cytokine
antagonist.

25   The invention further comprises the use of an ICAM-1, a CD18, a CD11b or
a VEGF antagonist in conjunction with a second antagonist. Genetic variability that
exists among various patient populations and/or additional mechanisms can warrant
administering more than one antagonist. Any combination of the above antagonists
can be used. For example, the present methods include administering an ICAM-1

30 antagonist, which is specific to a particular epitope of ICAM-1, and an additional
ICAM-1 antagonist, which is specific to a different epitope or genetic variation.

−16−

Similarly, an ICAM-1 antagonist can be administered with any one of the antagonists described herein. Administering a combination of antagonists to prevent the leukocyte adhesion to endothelial cells and/or leukocytes results in even more effective treatment of diabetic retinopathy or a more dramatic reduction in retinal

5   edema and/or ischemia. See Example 2 in which both a CD18 and CD11b antagonist was used to reduce leukocyte adhesion. Given the causal effect of leukocyte adhesion on retinal edema and/or ischemia, as proven by the data, administration of a CD18 and/or CD11b antagonist is expected to reduce retinal edema and/or retinal ischemia. The combination of antagonists can be administered

10   at substantially the same time, or sequentially, with suitable intervals between administration of the antagonists to confer the desired effect.

The invention also relates to decreasing or reducing the amount of ischemia and/or edema present in an individual by administering an effective amount of an ICAM-1 a CD18, a CD11b or a VEGF antagonist. Ischemia refers to tissue which

15   lacks proper or suitable blood flow. Ischemia refers to an inadequate circulation of blood flow which can be the result of a mechanical obstruction (e.g., trapped leukocyte) of the blood supply or damage to the blood supplying vessel which results in a reduction of the blood flow. Inadequate blood flow results in reduced tissue oxygenation. Hence, ischemia can be a function of leukostasis, and can be

20   measured by determining the density of trapped leukocytes, and other methods known in the art or developed in the future as described herein.

Edema refers to the build up of excess fluid caused by vasculature leakage (e.g., vascular permeability). Edema also refers to the build up or accumulation of fluid when the fluid is not timely or properly cleared. As described herein,

25   leukocytes become trapped in the capillaries in the conditions of reduced perfusion pressure (e.g., caused by constriction as seen in early stages of diabetes) or in the presence of an elevated adhesive stress between leukocytes and endothelium, endothelial swelling or narrowing of the capillary lumen by perivascular edema. The leukocyte build up can cause leakage from the blood vessel. Thus, edema can

30   be measured by determining the amount of retinal vascular albumin permeation, as referred to as "vascular permeability," as described herein.

–17–

The methods of treatment described herein include reducing or decreasing the amount of ischemia and/or edema by administering antagonist that inhibits leukocyte and endothelial cell interaction, as described herein. The decrease in ischemia is at least about 10% and can be greater, such as at least about 20%, 30%,
5   40%, 50% 60%, 70%, 80%, 90%, or 95%. The decrease in edema is at least about 10%, and can be greater, such as at least about 20%, 30%, 40%, 60%, 70%, 80%, 90%, and preferably at least about 95%.

The reduction or decrease of the retinal edema and/or retinal ischemia can be determined, as compared to a control, standard, or baseline. For example, a measure
10   of edema or ischemia can be made, in a mammal, prior to administering one of the compounds described herein, and one or more times subsequent to administration. A percentage change between two or more measurements, or a value reflecting the change in the measurements can be determined. The level of edema and/or ischemia can be quantified using methods known in the art, and a decrease, as compared with
15   a control, standard, or baseline, indicates successful treatment. The quantified amounts of edema and/or ischemia can be compared with a suitable control to determine if the levels are decreased. The sample to be tested can be compared with levels for the specific individual from previous time points (e.g., before having diabetic retinopathy, or during various phases of treatment for the diabetic
20   retinopathy), or with levels in normal individuals (e.g., an individual without the disease) or suitable controls. An individual who is being treated for diabetic retinopathy can be monitored by determining the levels of edema and/or ischemia at various time points. Such levels of edema and/or ischemia can be determined before treatment, during treatment, and after treatment. A decrease in the level of ischemia
25   and/or edema, as described herein, indicates successful treatment. Ischemia and/or edema can be measured using methods now known or those developed in the future. See Kohner E.M., et al. *Diabetic Retinopathy Metabolism*, 25:1985-1102 (1975). For example, ischemia and edema can be measured using a fluorescein angiogram or by measuring the vision loss in a patient. Edema can also be assessed by measuring
30   electrical signals or potential, visualizing the retina using a slit lamp, fluorescein angiogram, or by using a sensitive isotope dilution method.

−18−

Another aspect of the invention includes method for treating or preventing neovascularization. One of the more difficult problems in ophthalmology is treating the ocular surface abnormalities that accompany limbal cell injury. The limbus is a specialized tissue that marks the transition between cornea and conjunctiva. Stem

5   cells reside in this area and give rise to the normal corneal epithelium. When the limbus is sufficiently destroyed, an inflammatory corneal neovascularization ensues and a conjunctiva-like epithelium covers the cornea. The latter lacks the smoothness and cohesion of the normal corneal epithelium, making it optically inferior and prone to erosions. Corneal neovascularization. and the serum it delivers via leaky

10   vessels, supports the abnormal conjunctiva-like surface that covers the cornea. The selective injury of corneal vessels produced a reversion to a more normal corneal epithelial phenotype. Huang, A. *et al Ophthal.* 95:228 (1988). Unlike the experimental model, the laser injury of corneal vessels has not seen long-term success in humans. Thus, an effective treatment for the corneal neovascularization

15   that follows limbal injury has previously remained an elusive goal.

Corneal neovascularization secondary to limbal injury requires, in part, vascular endothelial growth factor (VEGF). VEGF induces intercellular adhesion molecule-1 (ICAM-1) expression in the vasculature of various tissues. Further, exogenous VEGF induces the adhesion of leukocytes to the endothelium of ocular

20   surface vessels, a process that can be partially blocked with anti-ICAM-1 antibodies. The effect of ICAM-1 and its common ligand the β_2 integrin CD18 was tested, on limbal injury-associated corneal neovascularization and inflammation in a pathophysiologically-relevant model.

Corneal neovascularization leads to vision loss in eyes that have undergone

25   extensive injury to the limbus. This situation characterizes a number of conditions, including traumatic alkali injury, Stevens Johnson syndrome and ocular cicatricial pemphagoid. Other conditions that involve neovascularization are diseases such as age-related macular degeneration, choroidal neovascularization, sickle cell retinopathy, retina vein occlusion, diabetic retinopathy, a condition associated with

30   limbal injury and a condition associated with increased neovascularization. To date, no treatments have proven effective at preventing the neovascularization associated

−19−

with these conditions. A pathophysiologically-relevant mouse model of limbal injury was utilized to test the role of CD18 and intercellular adhesion molecule-1 (ICAM-1) in the production of corneal neovascularization. The data described herein show that CD18 and ICAM-1 deficient mice have 39% (n=5, p=0.0054) and 33%

5   (n=5, p=0.013) less neovascularization, respectively, when compared to strain-specific normal controls. Corneal neutrophil counts were reduced by 66% (n=5, p=0.0019) and 39% (n=5, p=0.0016) in the CD18 and ICAM-1 deficient mice, respectively. Taken together, these data identify CD-18 and ICAM-1 as important mediators of the inflammation-associated neovascularization that follows limbal

10   injury. CD18 and ICAM-1 also serve as therapeutic targets for the treatment of the corneal neovascularization associated with limbal injury.

Hence, another embodiment of the invention includes methods of treating or preventing ocular (e.g., corneal, retinal or choroid) neovascularization in a mammal (e.g., an individual) by administering to the mammal a CD18 antagonist and an

15   ICAM-1 antagonist or CD18 antagonist. The inhibition of both CD18 and ICAM-1, or CD18, result in significantly less neovascularization, or as compared to a control, as defined herein. See Example 4.

Hence, the present methods utilize various forms of antagonists. An antagonist, as defined herein, means a compound that can inhibit, either partially or

20   fully, the binding of a leukocyte to an endothelial cell or to another leukocyte. An antagonist's biological activity also refers to a compound that can reduce or lessen the interaction between a leukocyte and an endothelial cell, or another leukocyte.

The terms, "antagonist" or "antibody," include proteins and polypeptides that are integrin (e.g., LFA-1, Mac-1 or p150,95) antagonists, integrin subunit (CD18,

25   CD11a or CD11b) antagonists, adhesion molecule (e.g., ICAM, PCAM or VCAM) antagonists, selectin(e.g., P-selectin, L-selectin or E-selectin) antagonists, or leukocyte adhesion-inducing cytokine antagonists or growth factor antagonists (e.g. antagonists to TNF-α, IL-1β, MCP-1 or VEGF). These terms also include proteins and polypeptides that have amino acid sequences analogous to the amino acid

30   sequence of the protein, as described herein, and/or functional equivalents thereof. These terms also encompass various analogues, homologues, or derivatives thereof.

−20−

Analogous amino acid sequences are defined to mean amino acid sequences with
sufficient identity to the antagonist's amino acid sequence so as to possess its
biological activity. For example, an analogous peptide can be produced with
"silent" changes in amino acid sequence wherein one, or more, amino acid residues

5   differ from the amino acid residues of the protein, yet still possess its biological
activity. Examples of such differences include additions, deletions, or substitutions
of residues of the amino acid sequence of the protein or polypeptide. Also
encompassed by these terms, are analogous polypeptides that exhibit greater, or
lesser, biological activity of the antagonist.

10   Antagonists also include antibody or antibody fragments, peptide mimetics
molecules, antisense molecules, ribozymes, aptamers (nucleic acid molecules), and
small molecule antagonists. Soluble forms of molecules (e.g., soluble ICAM) can
also act as an antagonist because it can bind to the leukocyte, thereby preventing the
membrane bound form from binding.

15   The term "antagonist" and "nucleic acid sequence" include homologues, as
defined herein. The homologous proteins and nucleic acid sequences can be
determined using methods known to those of skill in the art. Initial homology
searches can be performed at NCBI against the GenBank (release 87.0), EMBL
(release 39.0), and SwissProt (release 30.0) databases using the BLAST network

20   service. Altshul, SF, et al, *J. Mol. Biol.* 215: 403 (1990); Altschul, SF., *Nucleic
Acids Res.* 25:3389-3402 (1998). the teachings of both are incorporated herein by
reference. Computer analysis of nucleotide sequences can be performed using the
MOTIFS and the FindPatterns subroutines of the Genetics Computing Group (GCG,
version 8.0) software. Protein and/or nucleotide comparisons can also be performed

25   according to Higgins and Sharp (Higgins, D.G. and P.M. Sharp, "Description of the
method used in CLUSTAL," *Gene, 73*: 237-244 (1988)). Homologous proteins
and/or nucleic acid sequences are defined as those molecules with greater than 70%
sequences identity and/or similarity (e.g., 75%, 80%, 85%, 90%, or 95% homology).

Biologically active derivatives or analogs of the antagonists described herein

30   also include peptide mimetics. Peptide mimetics can be designed and produced by
techniques known to those of skill in the art. (see e.g., U.S. Patent Nos. 4,612,132;

5,643,873 and 5,654,276, the teachings of which are incorporated herein by reference). These mimetics can be based, for example, on the protein's specific amino acid sequence and maintain the relative position in space of the corresponding amino acid sequence. These peptide mimetics possess biological activity similar to

5   the biological activity of the corresponding peptide compound, but possess a "biological advantage" over the corresponding amino acid sequence with respect to one, or more, of the following properties: solubility, stability and susceptibility to hydrolysis and proteolysis.

Methods for preparing peptide mimetics include modifying the N-terminal

10   amino group, the C-terminal carboxyl group, and/or changing one or more of the amino linkages in the peptide to a non-amino linkage. Two or more such modifications can be coupled in one peptide mimetic molecule. Modifications of peptides to produce peptide mimetics are described in U.S. Patent Nos. 5,643,873 and 5,654,276, the teachings of which are incorporated herein by reference. Other

15   forms of the proteins, polypeptides and antibodies described herein and encompassed by the present invention, include those which are "functionally equivalent." This term, as used herein, refers to any nucleic acid sequence and its encoded amino acid which mimics the biological activity of the protein, polypeptide or antibody and/or functional domains thereof.

20   The term, "ICAM-1 antagonist" includes antagonists that directly (e.g., by inhibiting the ICAM-1 molecules itself) or indirectly inhibit ICAM-1 (e.g., by inhibiting a molecules that affects induction of ICAM-1 such as a VEGF antagonist or a TNF-α antagonist). Such antagonist are those which lead to a reduction in edema and/or ischemia. Antagonists also include other integrin antagonists (e.g., a

25   LFA-1 or p150,95 antagonists), selectin antagonists (e.g., P-selectin, E-selectin or L-selectin antagonist) and other adhesion molecule antagonists (e.g., ICAM-2, ICAM-3, PCAM or VCAM antagonist) or a leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist (an antagonist for TNF-1α , IL-1β, MCP-1 or VEGF).

30   An ICAM-1 antagonist is also a composition that inhibits the binding of ICAM-1 to a receptor or has the ability to decrease or affect the function of ICAM-1.

−22−

Such antagonists include antibodies to ICAM-1 (e.g., the IA29 antibody), antisense molecules that hybridize to nucleic acid which encodes ICAM-1. ICAM-1 antagonists also include ribozymes, aptimers, or small molecule inhibitors that are specific for ICAM-1 or the nucleic acid that encodes ICAM-1. Antagonists of

5 ICAM-1 include compounds which inhibit the binding between LFA-1 or Mac-1 and ICAM-1, or compounds that reduce the biological activity or function of ICAM-1. The biological activity of ICAM-1 refers to the ability to bind to LFA-1 or, in particular, to Mac-1, the ability to induce leukocyte adhesion, the ability to cause ischemia and/or the ability to cause edema.

10   The terms "antibody" or "immunoglobulin" refer to an immunoglobulin or fragment thereof having specificity to a molecule involved in leukocyte-leukocyte interaction or leukocyte-endothelium interaction. Examples of such antibodies include anti-integrin antibodies (e.g., antibodies specific for LFA-1, Mac-1 or p150,35), anti-integrin subunit antibodies (e.g., antibodies specific to CD18, CD11b

15 or a combination thereof), anti-selectin antibodies (e.g., antibodies specific to P-selection, E-selection and L-selectin), antibodies to leukocyte adhesion-inducing cytokine antagonists or growth factor antagonists (e.g., TNF-α , IL-1β, MCP-1 and VEGF antibodies), and adhesion molecule antibodies (e.g., ICAM-1, ICAM-2, ICAM-3, PCAM or VCAM antibodies). For example, the terms "ICAM-1

20 antibody," or "ICAM-1 immunoglobulin" refer to immunoglobulin or fragment thereof having specificity for ICAM-1.

        The term, "antibody" is also intended to encompass both polyclonal and monoclonal antibodies including transgenically produced antibodies. The terms polyclonal and monoclonal refer to the degree of homogeneity or an antibody

25 preparation and are not intended to be limited to particular methods of production. An antibody can be raised against an appropriate immunogen, such as an isolated and/or recombinant polypeptide (e.g., ICAM-1, CD18, CD11b, VEGF, or TNF-α) or portion thereof (including synthetic molecules such as synthetic peptides). In one embodiment, antibodies can be raised against an isolated and/or recombinant antigen

30 or portion thereof (e.g., a peptide) or against a host cell which expresses recombinant antigen or a portion thereof. In addition, cells expressing recombinant

-23-

antigen (e.g., ICAM-1, CD18, CD11b, VEGF, or TNF-α), such as transfected cells, can be used as immunogens or in a screening for an antibody which binds the receptor.

Preparation of immunizing antigen, and polyclonal and monoclonal antibody
5   production, can be performed using any suitable technique. A variety of methods
have been described (see e.g., Kohler *et al., Nature,* 256:495-497 (1975) and *Eur. J. Immunol. 6:* 511-519 (1976); Milstein *et al., Nature* 266:550-552 (1977); Koprowski *et al.,* U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual,* (Cold Spring Harbor Laboratory: Cold Spring
10   Harbor, NY); *Current Protocols In Molecular Biology,* Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.,* Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)).

Following immunization, anti-peptide antisera can be obtained from the immunized animal, and if desired, polyclonal antibodies can be isolated from the
15   serum. As described herein, purified recombinant proteins generated in *E. coli* were used to immunize rabbits to generate specific antibodies directed against the antigen. These antibodies recognize the recombinant protein expressed in *E. coli.* Monoclonal antibodies can also be produced by standard techniques which are well known in the art (Kohler and Milstein, *Nature* 256:495-497 (1975); Kozbar *et al.,*
20   *Immunology Today* 4:72 (1983); and Cole *et al.,* Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 (1985)). Generally, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0) with antibody producing cells. The antibody producing cell, preferably those of the spleen or lymph nodes, can be obtained from animals immunized with the antigen of
25   interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant
30   antibody from a library, by PCR, or which rely upon immunization of transgenic animals (e.g., mice) capable of producing a full repertoire of human antibodies (see

–24–

e.g., Jakobovits *et al., Proc. Natl. Acad. Sci. USA, 90*: 2551-2555 (1993); Jakobovits
et al., Nature, 362: 255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,545,806;
Surani *et al.*, U.S. Patent No. 5,545,807).

5   For example, the monoclonal antibody, IA29 can be used as described
herein. The IA29 antibody is specific for ICAM-1, and can be purchased from R and
D Systems, Minneapolis, Minnesota. Similarly, the anti-CD11a, anti-CD18, and
the anti-CD11b antibodies utilized in the experiments described herein are the WT.1
mAb, 6G2 mAb and the MRC OX-42 mAb. respectively, and can be obtained from
Serotec, Inc. (Raleigh, NC).

10   Functional fragments of antibodies, including fragments of chimeric,
humanized, primatized, veneered or single chain antibodies, can also be produced.
Functional fragments or portions of the foregoing antibodies include those which are
reactive with the antigen (e.g., ICAM-1, CD18, CD11b, VEGF, or TNF-α). For
example, antibody fragments capable of binding to the antigen or portion thereof,

15   including, but not limited to, Fv, Fab, Fab' and F(ab')$_2$ fragments are encompassed
by the invention. Such fragments can be produced by enzymatic cleavage or by
recombinant techniques. For instance, papain or pepsin cleavage can generate Fab
or F(ab')$_2$ fragments, respectively. Antibodies can also be produced in a variety of
truncated forms using antibody genes in which one or more stop codons has been

20   introduced upstream of the natural stop site. For example, a chimeric gene encoding
a F(ab')$_2$ heavy chain portion can be designed to include DNA sequences encoding
the CH$_1$ domain and hinge region of the heavy chain.

It will be appreciated that the antibody can be modified, for example, by
incorporation of or attachment (directly or indirectly (e.g., via a linker)) of a

25   detectable label such as a radioisotope, spin label, antigen (e.g., epitope label such as
a FLAG tag) or enzyme label, flourescent or chemiluminescent group and the like,
and such modified forms are included within the term "antibody."

A suitable antagonist is also an antisense molecule that can hybridize to the
nucleic acid which encodes the target polypeptide (e.g., ICAM-1, CD18, CD11b,

30   VEGF, or TNF-α). The hybridization inhibits transcription and/or synthesis of the
protein. Antisense molecules can hybridize to all, or a portion of the nucleic acid.

–25–

Producing such antisense molecules can be done using techniques well-known to those of skill in the art. For example, antisense molecules or constructs can be made using method known in the art. DeMesmaeker, Alain, et al., *Acc Chem. Res.* 28:366-374 (1995), Setlow, Jane K., *Genetic Engineering*, 20:143-151 (1998);

5   Dietz, Patent No. 5,814,500, filed October 31, 1996, entitled, "Delivery Construct for Antisense Nucleic Acids and Method of Use," the teachings all of which are incorporated by reference in their entirety. In particular, constructing an antisense molecule for an ICAM-1 antagonist is described in detail in WO 97/46671, entitled, "Enhanced Efficacy of Liposomal Anti-sense Delivery," the teachings of which are

10 incorporated by reference in their entirety. Additionally, developing an antisense molecule to inhibit a retinal disorder (e.g., retinopathy) is described in Robinson, G.S., *et al.*, *Proc. Natl. Acad. Sci.* 93:4851-4856 (1996).

Administration and dosages:

The terms "pharmaceutically acceptable carrier" or a "carrier" refer to any

15 generally acceptable excipient or drug delivery device that is relatively inert and non-toxic. The antagonist can be administered with or without a carrier. A preferred embodiment is to administer the antagonist (e.g., ICAM-1 antagonist) to the retinal area or the vasculature around or leading to the retina. Exemplary carriers include calcium carbonate, sucrose, dextrose. mannose, albumin, starch, cellulose,

20 silica gel, polyethylene glycol (PEG), dried skim milk, rice flour, magnesium stearate, and the like. Suitable formulations and additional carriers are described in Remington's Pharmaceutical Sciences, (17th Ed., Mack Pub. Co., Easton, PA), the teachings of which are incorporated herein by reference in their entirety. The antagonist can be administered systemically or locally (e.g., by injection or

25 diffusion).

Suitable carriers (e.g., pharmaceutical carriers) also include, but are not limited to sterile water, salt solutions (such as Ringer's solution), alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid esters,

30 hydroxymethylcellulose, polyvinyl pyrolidone, etc. Such preparations can be

sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, and/or aromatic substances and the like which do not deleteriously react with the active compounds. They can also be combined where desired with other active substances, e.g., enzyme inhibitors, to reduce metabolic degradation. A carrier (e.g., a pharmaceutically acceptable carrier) is preferred, but not necessary to administer an antagonist (e.g., an ICAM-1 antagonist).

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. In particular, carriers for parenteral administration include aqueous solutions of dextrose, saline, pure water, ethanol, glycerol, propylene glycol, peanut oil, sesame oil, polyoxyethylene-polyoxypropylene block polymers, and the like. Ampules are convenient unit dosages.

Preferably, the antagonist is administered locally to the eye, retinal area, choroid area or associated vasculature. The antagonist can also be administered to the cornea of the eye. The antagonist diffuses into the eye and contacts the retina or surrounding vasculature (e.g., eye drops, creams or gels).

One or more antagonists described herein can be administered. When administering more than one antagonist, the administration of the antagonists can occur simultaneously or sequentially in time. The antagonists can be administered before and after one another, or at the same time. Thus, the term "co-administration" is used herein to mean that the antagonists will be administered at times to reduce leukostasis, edema and/or ischemia. The methods of the present invention are not limited to the sequence in which the various antagonists are administered, so long as the antagonists are administered close enough in time to produce the desired effect. The methods also include co-administration with other drugs that used to treat retinopathy or other diseases described herein.

The compositions of the present invention can be administered intravenously, parenterally, orally, nasally, by inhalation, by implant, by injection,

–27–

or by suppository. The composition can be administered in a single dose or in more than one dose over a period of time to confer the desired effect.

The actual effective amounts of drug of the present invention can vary according to the specific drug being utilized, the particular composition formulated,
5   the mode of administration and the age, weight and condition of the patient, for example. As used herein, an effective amount of an ICAM antagonist is an amount of the drug which is capable of reducing the edema and/or ischemia levels. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations. (e.g. by means of an appropriate, conventional
10   pharmacological protocol).

EXEMPLIFICATION

Example 1: Prevention of Leukostasis and Vascular Leakage Diabetic Retinopathy via ICAM-1 Inhibition

Diabetic retinopathy is a leading cause of adult vision loss and blindness.
15   Much of the retinal damage that characterizes the disease results from retinal vascular leakage and non-perfusion. This study demonstrates that diabetic retinal vascular leakage and non-perfusion are temporally and spatially associated with retinal leukocyte stasis (leukostasis) in the rat model of streptozotocin-induced diabetes. Retinal leukostasis increases within days of developing diabetes and
20   correlates with the increased expression of retinal intercellular adhesion molecule-1 (ICAM-1). ICAM-1 blockade with a monoclonal antibody prevents diabetic retinal leukostasis (e.g., resulting in ischemia) and vascular leakage (e.g., resulting in edema) by 48.5% and 85.6%, respectively. These data identify the causal role of leukocytes in the pathogenesis of diabetic retinopathy and demonstrate the important
25   utility of ICAM-1 inhibition as a therapeutic strategy for the prevention of diabetic retinopathy.

While retinal vascular leakage and non-perfusion are recognized as two major complications of diabetes, their pathogenesis remains poorly understood. Leukocytes may be involved in the genesis of these complications. Diabetic

−28−

retinopathy is not generally considered an inflammatory disease, yet the retinal
vasculature of humans and rodents with diabetes mellitus contains increased
numbers of leukocytes. Many of these leukocytes are static. The causes and
consequences of this phenomenon are largely unknown. Intercellular adhesion

5   molecule-1 (ICAM-1) is a peptide that mediates leukocyte adhesion and
transmigration. ICAM-1 may be operative in the stasis observed in diabetic
retinopathy because ICAM-1 immunoreactivity is increased in the diabetic retinal
vasculature of humans. However, little is known about the direct pathogenetic role
of ICAM-1 in diabetic retinopathy. This study investigated the mechanisms of

10   diabetic retinal leukocyte stasis (leukostasis) and the role leukocytes play in the
development of two sight-threatening complications. vascular leakage and capillary
non-perfusion.

EXPERIMENTAL PROCEDURES
        Animals and Experimental Diabetes. Long-Evans rats weighing

15   approximately 200 g received a single 60 mg/kg injection of streptozotocin (Sigma,
St. Louis, MO) in 10 mM citrate buffer, pH 4.5, after an overnight fast. Control non-
diabetic animals received citrate buffer alone. Animals with blood glucose levels
greater than 250 mg/dl 24 hours later were considered diabetic. Blood pressure was
measured using a noninvasive cuff sensor and monitoring system (Ueda Electronics,

20   Tokyo, Japan). Blood anticoagulated with EDTA was drawn from the abdominal
aorta of each rat after the experiment. The blood sample was analyzed using a
hematology analyzer. The rats were fed on standard laboratory chow and were
allowed free access to water in an air-conditioned room with a 12-hour light–12-
hour dark cycle until they were used for the experiments.

25   Acridine Orange Leukocyte Fluorography (AOLF) and Fluorescein
Angiography. Leukocyte dynamics in the retina were studied with AOLF
(Miyamoto, K., et al., Invest. Ophthalmol. Vis. Sci., 39:2190-2194 (1998);
Nishiwaki, H., et al., Invest. Ophthalmol. Vis. Sci., 37:1341-1347 (1996); Miyamoto,
K., et al., Invest. Ophthalmol. Vis. Sci., 37:2708-2715 (1996)). Intravenous injection

30   of acridine orange causes leukocytes and endothelial cells to fluoresce through the

–29–

non-covalent binding of the molecule to double stranded nucleic acid. When a
scanning laser ophthalmoscope is utilized, retinal leukocytes within blood vessels
can be visualized *in vivo*. Twenty minutes after acridine orange injection, static
leukocytes in the capillary bed can be observed. Immediately after observing and
5   recording the static leukocytes, fluorescein angiography was performed to study the
relationship between static leukocytes and retinal vasculature.

Twenty-four hours before AOLF and fluorescein angiography were
performed, all rats had a heparin-lock catheter surgically implanted in the right
jugular vein for the administration of acridine orange or sodium fluorescein dye. The
10 catheter was subcutaneously externalized to the back of the neck. The rats were
anesthetized for this procedure with xylazine hydrochloride (4 mg/kg) (Phoenix
Pharmaceutical, St. Joseph, MO) and ketamine hydrochloride (25 mg/kg) (Parke-
Davis, Morris Plains, NJ). Immediately before AOLF, each rat was again
anesthetized, and the pupil of the left eye was dilated with 1% tropicamide (Alcon,
15 Humancao, Puerto Rico) to observe leukocyte dynamics. A focused image of the
peripapillary fundus of the left eye was obtained with a scanning laser
ophthalmoscope (SLO; Rodenstock Instrument, Munich, Germany). Acridine orange
(Sigma, St. Louis, MO) was dissolved in sterile saline (1.0 mg/ml) and 3 mg/kg was
injected through the jugular vein catheter at a rate of 1 ml/min. The fundus was
20 observed with the SLO using the argon blue laser as the illumination source and the
standard fluorescein angiography filter in the 40° field setting for 1 minute. Twenty
minutes later, the fundus was again observed to evaluate leukostasis in the retina.
Immediately after evaluating retinal leukostasis, 20 µl of 1% sodium fluorescein dye
was injected into the jugular vein catheter. The images were recorded on a videotape
25 at the rate of 30 frames/sec. The video recordings were analyzed on a computer
equipped with a video digitizer (Radius, San Jose, CA) that digitizes the video
image in real time (30 frames/sec) to 640 x 480 pixels with an intensity resolution of
256 steps. For evaluating retinal leukostasis, an observation area around the optic
disc measuring ten disc diameters in diameter was determined by drawing a polygon
30 surrounded by the adjacent major retinal vessels. The area was measured in pixels
and the density of trapped leukocytes was calculated by dividing the number of

–30–

trapped leukocytes, which were recognized as fluorescent dots, by the area of the observation region. The densities of leukocytes were calculated generally in eight peripapillary observation areas and an average density was obtained by averaging the eight density values.

5   Isotope Dilution Technique. Vascular leakage was quantified using an isotope dilution technique based on the injection of bovine serum albumin (BSA) labeled with two different iodine isotopes, ^{125}I and ^{131}I. Briefly, purified monomer BSA (1 mg) was iodinated with 1 mCi of ^{131}I or ^{125}I using the iodogen method. Polyethylene tubing (0.58 mm internal diameter) was used to cannulate the right

10   jugular vein and the left or right iliac artery. The tubing was filled with heparinized saline. The right jugular vein cannula was used for tracer injection. The iliac artery cannula was connected to a one ml syringe attached to a Harvard Bioscience model PHD 2000 constant-withdrawal pump preset to withdraw at a constant rate of 0.055 ml/min. At time 0, $[^{125}I]BSA$ (50 million cpm in 0.3 ml of saline) was injected into

15   the jugular vein and the withdrawal pump started. At the eight-minute mark, 0.2 ml (50 million cpm) of $[^{131}I]BSA$ was injected. At the ten-minute mark, the heart was excised, the withdrawal pump was stopped, and the retina was quickly dissected and sampled for g-spectrometry. Tissue and arterial samples were weighed and counted in a g-spectrometer (Beckman 5500, Irvine, CA). The data were corrected for

20   background and a quantitative index of $[^{125}I]BSA$ tissue clearance was calculated as previously described and expressed as μg plasma x g tissue wet weight^{-1} x min^{-1}. Briefly, $[^{125}I]$ BSA tissue activity was corrected for $[^{125}I]$ BSA contained within the tissue vasculature by multiplying $[^{125}I]BSA$ activity in the tissue by the ratio of $[^{125}I]BSA/[^{131}I]BSA$ in the arterial plasma sample obtained at the end of the

25   experiment. The vascular-corrected $[^{125}I]BSA$ activity was divided by the time-averaged $[^{125}I]BSA$ plasma activity (obtained from a well-mixed sample of plasma taken from the withdrawal syringe) and by the tracer circulation time (10 minutes) and then normalized per gram tissue wet weight.

Ribonuclease Protection Assay. The retinas were gently dissected free and

30   cut at the optic disc after enucleation, and frozen immediately in liquid nitrogen. Total RNA was isolated from rat retinas according to the acid guanidinium

−31−

thiocyanate-phenol-chloroform extraction method. A 425-base pair EcoRI/BamHI
fragment of rat ICAM-1 cDNA was prepared by reverse transcription-polymerase
chain reaction and cloned into pBluescript II KS vector. A 472 nucleotide antisense
riboprobe was prepared by *in vitro* transcription (Promega, Madison, WI) of
5   linearized plasmid DNA with T7 RNA polymerase in the presence of $[^{32}P]$dUTP.
The sequence of the cloned cDNA was verified by DNA sequencing. Twenty
micrograms of total cellular RNA were used for ribonuclease protection assays. All
samples were simultaneously hybridized with an 18S riboprobe (Ambion, Austin,
TX) to normalize for variations in loading and recovery of RNA. Protected
10 fragments were separated on a gel of 5% acrylamide, 8M urea, 1x Tris-borate-
EDTA, and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale,
CA).

        ICAM-1 Blockade. Twenty four hours following streptozotocin injection,
confirmed diabetic animals received intraperitoneal injections of 3 mg/kg or 5
15 mg/kg rat ICAM-1 neutralizing antibody (1A29; R&D Systems, Minneapolis MN)
or 5 mg/kg normal mouse IgG1 (R&D Systems) in sterile phosphate buffered saline.
The animals were treated three times per week. Retinal leukostasis and vascular
leakage were studied one week following diabetes induction.

        Statistical Analysis. All results are expressed as means ± SD. The data were
20 compared by analysis of variance (ANOVA) with post-hoc comparisons tested using
Fisher's protected least significant difference (PLSD) procedure. Differences were
considered statistically significant when P values were less than 0.05.

RESULTS AND DISCUSSION

        Time-Course Changes of Retinal Leukostasis and Vascular Leakage after
25 Diabetes Induction. Retinal leukostasis was quantified in Long-Evans rats. Diabetic
rats, like humans with diabetes, develop retinal non-perfusion and increased vascular
permeability. Figure 1 shows the time course of diabetic retinal leukostasis and
vascular leakage. In Figure 1A, leukostasis was serially quantified using AOLF.
Non-diabetic animals (day 0) and animals with streptozotocin-induced diabetes of
30 varying duration were studied. Using AOLF, a time course analysis showed that

−32−

retinal leukostasis increased 1.9-fold as early as three days following diabetes
induction (n=5, p<0.05) (Figure 1A). After one week of diabetes, retinal leukostasis
was 3.2-fold higher than in non-diabetic controls (n=5, p<0.0001). This finding
remained unchanged in degree for three additional weeks (n=5, p<0.0001) (Figure

5 1A). Reliable leukostasis quantitation beyond the four-week time point was
precluded by cataract formation.

Leukocyte adhesion to endothelial cells can trigger the disorganization of
endothelial cell adherens and tight junctions and vascular leakage. To determine if
diabetic retinal leukostasis was correlated with blood-retinal barrier breakdown,

10 retinal albumin permeation was quantified (Figure 1B). In Figure 1B, radioactive
albumin permeation into retinal tissue was quantitated at the same time points using
the isotope dilution technique. Retinal albumin permeation characterizes human and
rodent diabetic retinopathy and can be sensitively quantified using the isotope
dilution technique (Tilton, R. G., *et al.*, *Diabetes* 42:221-232 (1993); Tilton, R. G.,

15 *et al.*, *J. Clin. Invest.* 99:2192-2202 (1997); and Vinores, S. A., *et al.*, *Am. J. Pathol.*,
134:231-235 (1989). A time course analysis in diabetic rats revealed a 2.9-fold
(n=8, p<0.0001) and 10.7-fold (n=8, p<0.0001) increase in albumin permeation
following one and four weeks of diabetes (Figure 1B). The breakdown of the blood-
retinal barrier followed the onset of diabetes-associated retinal leukostasis.

20 Leukocyte-Induced Non-perfusion and Reperfusion in Retinal Capillaries.
To further characterize the diabetic retinal leukostasis, serial AOLF and fluorescein
angiography studies were performed. Figure 2 shows that static leukocytes are in
flux, block capillary flow and transmigrate. Serial AOLF of static leukocytes in the
same retinal area after seven (Figure 2A) and eight (Figure 2C) days of diabetes

25 shows their complete replacement within a 24 hour period. The arrow points to a
static leukocyte (Figures 2A and 2B) that appears to have transmigrated (Figure 2B).
One day later, AOLF and fluorescein angiography show that the leukocyte has
disappeared (Figures 2C and 2D). The arrowhead shows a patent capillary (Figure
2B) that subsequently becomes obstructed by a static leukocyte 24 hours later

30 (Figures 2C and 2D). These studies revealed that the individual leukocytes observed
with AOLF are in flux, even though the overall degree of leukostasis is constant

–33–

(Figure 2). The static retinal leukocytes observed seven days following the induction of diabetes are topographically distinct from those observed 24 hours later. Furthermore, a fraction of the leukocytes are in the extravascular space, a result consistent with their rapid transmigration following dye labeling.

5   Fluorescein angiography and AOLF were also used to study retinal non-perfusion. These studies identified static leukocytes directly associated with areas of downstream non-perfusion (Figures 2 and 3). Figure 3 shows leukocyte-induced non-perfusion and reperfusion. Serial studies were completed one (Figures 3A and 3B), two (Figures 3C and 3D) and four (Figures 3E and 3F) weeks following

10   diabetes induction using both AOLF (Figures 3A, 3C, and 3E) and fluorescein angiography (Figures 3B, 3D, and 3F). The arrow shows a patent capillary (Figure 3B) that subsequently becomes occluded downstream from a static leukocyte (Figures 3C and 3D), and then opens up when the leukocyte disappears (Figures 3E and 3F). The arrowhead shows a patent capillary (Figure 3B) that becomes

15   occluded downstream from a static leukocyte (Figures 3C and 3D) and then remains closed after the leukocyte has disappeared (Figures 3E and 3F). The non-perfused capillaries were patent prior to the onset of the leukostasis, indicating a causal relationship. As the leukocyte(s) disappeared, the capillaries either reperfused or remained closed (Figure 3). Reperfusion has been observed in human diabetic

20   retinopathy, but the mechanism, until now, has remained unexplained.

           ICAM-1 Gene Expression in Diabetic Retina. To determine if retinal ICAM-1 expression increases in association with diabetic retinal leukostasis, ICAM-1 mRNA levels were quantified using the ribonuclease protection assay. Figure 4 shows ICAM-1 gene expression in diabetic retina. Figure 4A shows results from a

25   ribonuclease protection assay, which demonstrates that retinal ICAM-1 levels were significantly increased seven days following diabetes induction. Each lane shows the signal from the two retinas of a single animal. The lane labeled "Probes" shows a hundred-fold dilution of the full-length ICAM-1 and 18S riboprobes. The lanes labeled "RNase - (0.1)" and "RNase - (0.01)" show the ten-fold and hundred-fold

30   dilutions, respectively, of the full-length riboprobes without sample or RNase. When normalized to 18S RNA, the retinal ICAM-1 levels after seven days of diabetes were

-34-

2.2-fold higher (n=4, p<0.05) than in the non-diabetic controls (Figure 4B). Retinas analyzed three days following diabetes induction demonstrated that retinal ICAM-1 mRNA levels were 1.5-fold higher than non-diabetic controls, but this increase was not statistically significant (n=5, p>0.05) (Figure 4). After one week of diabetes, the

5   retinal ICAM-1 levels were 2.2-fold greater, a significant increase when compared to non-diabetic controls (n=4, p<0.05). The ICAM-1 increase coincided temporally with the development of diabetic retinal leukostasis and blood-retinal barrier breakdown.

        An Anti-ICAM-1 Monoclonal Antibody (mAb) Prevents Leukostasis and

10   Vascular Leakage in Diabetic Retina. To assess whether ICAM-1 mediates diabetic retinal leukostasis, a well characterized ICAM-1 neutralizing antibody (1A29) was used for *in vivo* adhesion blockade experiments. Tamatani, T. *et al., Int. Immunol.* 2, 165-171 (1990); Kawasaki, K., *et al., J. Immunol.* 150, 1074-1083 (1993); Kelly, K. *et al., Proc. Natl. Acad. Sci.* USA 91, 812-816 (1994). Animals received

15   either 3 or 5 mg/kg intraperitoneal injections of the ICAM-1 antibody three times weekly. Control diabetic animals received an equivalent amount of a non-immune isotype control antibody. All animals were analyzed one week following diabetes induction. The results showed that the ICAM-1 antibody blocked diabetes-induced leukostasis by 40.9% (3 mg/kg, n=5, p<0.01) and 48.5% (5 mg/kg, n=5, p<0.001)

20   (Figures 5 and 6A). The peripheral leukocyte counts at one week increased by 40% (5 mg/kg, n=5, p<0.05) compared to the control antibody treated animals, a result consistent with successful systemic ICAM-1 blockade (Table 1). Body weight, plasma glucose and blood pressure were unchanged in all diabetic groups (Table 1).

Table 1:

Characteristics of control, diabetic, mouse IgG1-treated diabetic, and anti-ICAM-1

mAb-treated diabetic rats

	Control	Diabetes	Diabetes + 5mg/kg mouse IgG1	Diabetes+anti-ICAM-1 mAb	
				3mg/kg	5mg/kg
n	6 CR	7	5	5	5
Body weight (g)	271±12	240±12*	235±9*	238±6*	239±12*
Plasma glucose (mg/dl)	123±19	332±35*	316±61*	351±83*	373±68*
Blood Pressure (mmHg)	111±6	104±12	109±14	105±9	105±10
Leukocyte count (x10^3/μl)	6.1±1.6	5.0±1.5♦	5.3±0.8♦	6.9±1.4	7.4±2.3

Values are means ±SD. *$P<0.0001$ vs. control rats; ♦$P<0.05$ vs. 5 mg/kg anti-
ICAM-1 mAb-treated diabetic rats. All results are expressed as means ± SD.
Unpaired groups of two were compared using two sample t-test or two sample t-test
with Welch's correction. To compare three or more groups, analysis of variance was
followed by the post hoc test with Fisher's PLSD procedure. Differences were
considered statistically significant when P values were less than 0.05.

The effect of the ICAM-1 inhibition on blood-retinal barrier breakdown was
tested using the same antibody. Animals receiving 3 and 5 mg/kg of the anti-ICAM-
1 antibody had 63.5% (3 mg/kg, n=4, p<0.0001) and 85.6% (5 mg/kg, n=4,
p<0.0001) less retinal albumin permeation at one week (Figure 6B). The results
suggest that the ICAM-1-dependent component of the leukostasis is largely
responsible for the blood-retinal barrier breakdown.

Example 2:   Integrin-mediated neutrophil adhesion and retinal leukostasis in
diabetes

INTRODUCTION:

Leukocyte-endothelial cell interactions in tissues are mediated by adhesion
molecules expressed on the surface of leukocytes and endothelial cells.
Immunoglobulin superfamily molecules such as ICAM-1 are expressed on
endothelial cells and bind to β_2-integrins expressed on leukocytes. The integrins are
transmembrane receptors that consist of noncovalently bound heterodimers
composed of α- and β-chains. The β_2-integrins are operative in leukocyte adhesion
and include LFA-1 (lymphocyte function associated antigen, CD11a/CD18), Mac-1
(leukocyte adhesion receptor, CD11b/CD18) and p150/95 (CD11c/CD18). Each of
the β_2-integrins has a common β-chain in combination with a unique α-chain. CD18
is required for the firm attachment of healthy human neutrophils to human umbilical
vein endothelial cells.

In vivo studies from our laboratory have investigated the role of leukocytes
in diabetic retinopathy. Utilizing acridine orange leukocyte fluorography, the
density of static leukocytes in the retinas of streptozotocin-induced diabetic rats was
demonstrated to be increased. Retinal leukocyte stasis (leukostasis) was observed
within three days of diabetes induction, and was temporally and spatially correlated
with capillary non-perfusion and blood-retinal barrier breakdown. The onset of
retinal leukostasis coincided with the upregulation of retinal ICAM-1 expression.
Causality was demonstrated when an anti-ICAM-1 antibody prevented the diabetes-
associated increases in retinal leukostasis and vascular leakage by 48.5% and 85.6%,

–37–

respectively. However the identities and bioactivities of the neutrophil adhesion
molecules mediating diabetic retinal leukostasis are less well understood.

      The aim of the current study was to investigate in greater detail the role of
neutrophils in early diabetic retinal leukostasis. A time point of one week of
5   diabetes was chosen in this study because steady-state increases in diabetic retinal
leukostasis and ICAM-1 expression are achieved in one week. Since adhesion can
occur in the absence of increased adhesion molecule expression, both adhesion
molecule expression and bioactivity were examined. Finally, the role of CD18 in the
development of diabetic retinal leukostasis was examined *in vivo* using acridine
10 orange leukocyte fluorography and neutralizing anti-CD18 F(ab')$_2$ fragments.

METHODS:

      Diabetes was induced in Long Evans rats with streptozotocin. The
expression of the surface integrin subunits CD11a, CD11b, and CD18 on rat
neutrophils isolated from peripheral blood was quantitated with flow cytometry. *In*
15 *vitro* neutrophil adhesion was studied using quantitative endothelial cell-neutrophil
adhesion assays. The adhesive role of the integrin subunits CD11a, CD11b and
CD18 was tested using specific neutralizing monoclonal antibodies. CD18
bioactivity was blocked *in vivo* with anti-CD18 F(ab')$_2$ fragments and the effect on
retinal leukocyte adhesion was quantitated with acridine orange leukocyte
20 fluorography (AOLF).

Animals

      Male Long-Evans rats weighing approximately 200 g were used for these
experiments. The rats were fed standard laboratory chow and allowed free access to
water in an air-conditioned room with a 12-hour light-12-hour dark cycle.

25 Induction of diabetes

      Rats received a single 60 mg/kg intraperitoneal injection of streptozotocin
(Sigma, St. Louis, MO) in 10mM sodium citrate buffer, pH 4.5, after an overnight
fast. Control non-diabetic animals received citrate buffer alone. Animals with blood

-38-

glucose levels greater than 250 mg/dl 24 hours after injection were considered diabetic. All experiments were performed one week following the induction of diabetes.

Monoclonal antibodies and F(ab')2 fragments

5   The monoclonal antibodies (mAb) were murine in origin and were used as purified IgG. For the *in vitro* studies, mAbs WT.1 (anti-rat CD11a), 6G2 (anti-rat CD18), and MRC OX-42 (anti-rat CD11b) were obtained from Serotec Inc. (Raleigh, NC). FITC-conjugated mouse IgG_1 mAb isotype control was obtained from PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated

10   goat anti-mouse IgG_1 Ab was obtained from Caltag Laboratories (Burlingame, CA). For the *in vivo* studies, WT.3 anti-rat LFA-1 beta chain (CD18) F(ab')$_2$ fragments were obtained from Seikagaku America (Division of Associates of Cape Cod, Inc., Falmouth, MA). Purified mouse anti-human IgG F(ab')$_2$ fragments were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

15   Flow cytometry

        The surface expression of CD11a, CD11b, and CD18 on rat neutrophils was determined using flow cytometry as previously described. Allport JR, *et al., J Immunol., 158*:4365-4372 (1997). Briefly, whole blood anticoagulated with EDTA was obtained from the hearts of rats anesthetized with inhaled isofluorane.

20   Leukocytes were isolated by dextran sedimentation and hypotonic lysis of contaminating erythrocytes. Aliquots of 5 x 10^5 cells in 100 µl RPMI 1640 medium (BioWhittaker, Walkersville, MD) containing 5% fetal bovine serum (RPMI-5%) were incubated on ice for 10 min. The tubes were centrifuged at 400 x g for 5 min at 4°C. The cell pellets were resuspended in 100 µl RPMI-5% containing 20 µg/ml

25   primary mAb to CD11a, CD11b, CD18 or isotype control and incubated for 45 min on ice. Primary mAb were detected with FITC-conjugated goat anti-mouse IgG_1 Ab as previously detailed. The fluorescence of 10^4 cells was measured on a FACScan (Becton Dickinson, San Jose, CA). Neutrophils were manually gated on the basis of

−39−

their characteristic forward and side light scattering properties. The surface

expression is presented as the mean channel fluorescence on a logarithmic scale.

Endothelial cell-neutrophil adhesion assays

        Peripheral blood was obtained from rats anesthetized with inhaled

5   isofluorane via heart puncture with a 16-gauge EDTA flushed needle. Neutrophils

were isolated from whole blood by density gradient centrifugation with NIM•2™

(Neutrophil Isolation Media; Cardinal Associates, Santa Fe, NM) according to the

manufacturer's instructions. Preparations contained > 94% neutrophils as

determined by eosin and methylene blue staining (Leukostat staining system;

10   Fischer Scientific, Pittsburgh, PA). There was no red blood cell contamination. The

cells were used immediately after collection.

        The adhesion of unstimulated neutrophils to confluent monolayers of rat

prostate endothelial cells (RPEC) was determined under static conditions as

previously described. (Luscinskas FW, et al., J Immunol., 149:2163 (1992); Kiely

15   JM, et al., "Methods in Molecular Biology, Adhesion Protein Protocols,"

Leukocyte-endothelial monolayer adhesion assay (static conditions), 131-136

(1999). RPEC were obtained from the American Type Culture Collection (ATCC;

Manassas, VA) and cultured in Eagle's minimum essential media (ATCC)

supplemented with 5% fetal bovine serum (FBS; GIBCO, Gaithersburg, MD) and

20   0.3 ng/ml porcine intestinal heparin (Sigma, St. Louis, MO). RPEC were grown to

confluence on tissue culture-treated plastic microtiter 96-well plates, stimulated for

24-hours with 30 ng/ml recombinant human TNF-α (Genzyme Corp., Cambridge,

MA), and incubated for 15 minutes with RPMI-5%. TNF-α stimulation of ICAM-1

surface expression was utilized for all experiments. Neutrophils were resuspended at

25   2×10^6 cells/ml in RPMI-5% and incubated for 10 min at 37°C with 1 μM of the

fluorescent marker, 2',7'-bis-(2-carboxyethyl)-5 (and 6) carboxyfluorescein,

acetoxymethyl ester (Molecular Probes, Eugene, OR) in DMSO (vehicle).

Fluorescent labeled neutrophils were washed once and then incubated in RPMI-5%

alone or RPMI-5% with a saturating concentration of mAb (30 μg/ml) to CD11a,

30   CD11b, or CD18 for 10 min at room temperature. The neutrophils were washed and

then incubated (2×10^6 neutrophils/ml, 50 μl per well) with RPEC for 10 min at
37°C. Non-adherent cells were removed and the content of the wells lysed with
10mM Tris-HCl, pH 8.4 containing 0.1% SDS. Fluorescence was determined in a
microtiter plate fluorimeter (excitation 485 nm, emission 530-540 nm) and the

5   adhesion reported as the number of adherent neutrophils/mm^2.

Acridine orange leukocyte fluorography (AOLF)

Leukocyte dynamics in the retina were studied with AOLF. (Miyamoto, K.,
et al., "In vivo demonstration of increased leukocyte entrapment in retinal
microcirculation of diabetic rats," *Invest Opthalmol Vis Sci.*, *39*:2190-2194 (1998);

10 Miyamoto, K., *et al.*, *Proc Natl Acad Sci USA.*, *96(19)*:10836-41 (1999); Nishiwaki,
H., *et al.*, *Invest Ophthalmol Vis Sci.*, *36*:123-130 (1995); Nishiwaki, H., *et al.*,
Invest Ophthalmol Vis Sci., *37*:1341-1347 (1997)). Rats were anesthetized with 4
mg/kg xylazine hydrochloride (Phoenix Pharmaceutical, St. Joseph, MO) and 25
mg/kg ketamine hydrochloride (Parke-Davis, Morris Plains, NJ). The day before

15 leukocyte dynamics were observed, a heparin-lock catheter was surgically implanted
in the right jugular vein of each rat. The catheter was subcutaneously externalized to
the back of the neck. Rats received intravenous injections of 5 mg/kg anti-rat beta
chain (CD18, WT.3) $F(ab')_2$ fragments or 5 mg/kg anti-human IgG isotype control
$F(ab')_2$ fragments in sterile phosphate buffered saline 24 hours before AOLF was

20 performed. The experiments were carried out in a masked fashion.

Immediately before AOLF, each rat was again anesthetized, and the pupil of
the left eye was dilated with 1% tropicamide (Alcon, Humancao, Puerto Rico) to
observe leukocyte dynamics. A focused image of the peripapillary fundus of the left
eye was obtained with a scanning laser ophthalmoscope (SLO; Rodenstock

25 Instruments, Munich, Germany). Acridine orange (Sigma, St. Louis, MO) was
dissolved in sterile saline (1.0 mg/ml) and 3 mg/kg was injected through the jugular
vein catheter at a rate of 1 ml/min. The fundus was observed with the SLO using the
argon blue laser as the illumination source and the standard fluorescein angiography
filter in the 40° field setting for 1 min. Twenty min later, the fundus was again

30 observed to evaluate leukostasis in the retina. The images were recorded on

-41-

videotape at the rate of 30 frames/sec. The video recordings were analyzed on a computer equipped with a video digitizer (Radius, San Jose, CA) that digitizes the video image in real time (30 frames/sec) to 640 x 480 pixels with an intensity resolution of 256 steps. For evaluating retinal leukostasis, an observation area

5   around the optic disc measuring five disc diameters in radius was determined by drawing a polygon bordered by the adjacent major retinal vessels. The density of trapped leukocytes was calculated by dividing the number of static leukocytes (recognized as fluorescent dots) by the area of the observation region (in pixels). The density of static leukocytes was calculated in 8-10 peripapillary observation areas

10   and an average density ($x10^{-5}$ cells/pixel^2) was obtained.

Blood pressures and heart rates were measured using a noninvasive cuff sensor and monitoring system (Ueda Electronics, Tokyo, Japan). Blood anticoagulated with EDTA was drawn from the abdominal aorta of each rat after the experiment to determine the leukocyte count using a hematology analyzer. The

15   leukocyte count was determined using a hematology analyzer.

Statistical analysis

All results are expressed as means ± SD. The data were compared by analysis of variance (ANOVA) with post-hoc comparisons tested using Fisher's protected least significant difference (PLSD) procedure. Differences were

20   considered statistically significant when p values were less than 0.05.

RESULTS

Neutrophil CD11a, CD11b, and CD18 surface integrin levels were 62% (n=5, p=0.006), 54% (n=5, p=0.045) and 38% (n=5, p=0.009) greater in diabetic vs. non-diabetic animals, respectively. Seventy-five percent more neutrophils from

25   diabetic vs. non-diabetic animals adhered to rat endothelial cell monolayers (n=6, p=0.02). Pre-treatment of leukocytes with either anti-CD11b or anti-CD18 antibodies lowered the proportion of adherent diabetic neutrophils by 41% (n=6, p=0.01 for each treatment), while anti-CD11a antibodies had no significant effect

—42—

(n=6, p=0.5). *In vivo,* systemic administration of anti-CD18 F(ab')$_2$ fragments decreased diabetic retinal leukostasis by 62% (n=5, p=0.001).

Increased surface integrin expression on diabetic neutrophils

5   Integrin expression was measured on the surface of neutrophils from normal and diabetic rats. As shown in Table 2, the flow cytometric analyses demonstrated statistically significant increases in the diabetic leukocyte CD11a, CD11b, and CD18 levels, as evidenced by the increases in mean channel fluorescence. Neutrophil CD11a, CD11b, and CD18 levels were 62% (n=5, p=0.006), 54% (n=5, p=0.045), and 38% (n=5, p=0.009) greater, respectively, on the one week-diabetic

10   leukocytes vs. the non-diabetic leukocytes. Integrin expression was similarly increased on two week-diabetic neutrophils with CD11a, CD11b, and CD18 levels being 53%, 24%, and 38% greater, respectively.

Table 2. Flow-cytometric analysis of integrin molecule expression on neutrophils.

	Control	Diabetes	Diabetes + control F(ab;)$_2$	Diabetes + anti-CD18 F(ab')$_2$
n	5	5	5	5
Body Weight (g)	268±10	236±4*	233±7*	237±17*
Plasma glucose (mg/dl)	122±21	327±40*	357±60*	351±28*
Blood pressure (mmHg)	110±7	106±13	103±8	103±7
Leukocyte count (x10^3 μL)	6.4±1.4	4.9±1.6†	5.0±1.3	6.7±0.9

Values are means ± SD.

*P< 0.001 vs. control rats

†P< 0.05 vs. anti-CD18 F(ab')^2-treated diabetic rats.

Diabetic neutrophils exhibit increased adhesion to TNFα-activated
endothelial cell monolayers *in vitro*

The functional adhesion of purified neutrophils to cultured endothelial cell
monolayers was investigated. Adhesion assays were performed by adding diabetic or
non-diabetic neutrophils to TNF-α-stimulated rat endothelial cell monolayers under
static conditions. TNF-α was added to maximize endothelial cell ICAM-1
expression. Preliminary experiments demonstrated a 2.7-fold increase in endothelial
cell ICAM-1 expression with TNF-α (n=4, p<0.0001). Figure 7 shows that adhesion
of control and diabetic rat neutrophils to confluent TNF-activated endothelial cell
monolayers under static conditions. Neutrophils isolated from diabetic rats
demonstrated significantly increased adhesion to rat endothelial cell monolayers.
Figure 7 shows that adhesion of control and diabetic rat neutrophils to confluent
TNF-activated rat endothelial cell monolayers under static conditions. Neutrophils
isolated from diabetic rats demonstrated significantly increased adhesion to rat
endothelial cell monolayers.

As shown in Figure 7, 75% more neutrophils from the diabetic rats adhered to the
endothelial cell monolayers than neutrophils isolated from non-diabetic rats (n=6,
p=0.02).

The β$_2$-integrin molecules mediating neutrophil adhesion *in vitro* were
examined. Figure 8 shows the effect of anti-integrin antibodies on neutrophil
adhesion *in vitro*. Neutrophils were pre-incubated with anti-CD11a, anti-CD11b,
anti-CD18 (30 µg/ml of each mAb), or an equimolar mixture of anti-
CD11a/CD11b/CD18 antibodies prior to their use in the adhesion studies. In a
representative experiment shown in Figure 8, untreated diabetic neutrophils
exhibited increased adhesion to TNFα-activated endothelial cell monolayers under
all treatment conditions. Pretreatment with anti-CD11b or anti-CD18 antibodies
each decreased diabetic neutrophil adhesion by 41% (n=6, p=0.01 for each
treatment). In contrast, pretreatment with the anti-CD11a antibody did not
significantly affect diabetic neutrophil adhesion (n=6, p=0.5 vs. untreated diabetic
neutrophils). Moreover, treatment with an equimolar mixture of anti-CD11a, anti-
CD11b, and anti-CD18 monoclonal antibodies significantly reduced diabetic

−45−

neutrophil adhesion by 72% (n=6, p<0.0001 vs.untreated diabetic neutrophils). Non-
diabetic neutrophil adhesion was also reduced with the anti-CD11a, anti-CD11b and
anti-CD18 antibodies, as well as with the anti-CD11a/CD11b/CD18 antibody
cocktail. The decreases were 39%, 49%, 53%, and 52%, respectively (n=6, p<0.05
5   for each treatment vs. untreated non-diabetic neutrophils).

In vivo CD8 blockade decreases leukostasis in diabetic rat retinas

        Retinal leukostasis in living animals was measured with AOLF. Intravenous
injection of acridine orange causes leukocytes and endothelial cells to fluoresce
through the non-covalent binding of the molecule to double stranded DNA. When a
10   scanning laser ophthalmoscope is utilized, retinal leukocytes within blood vessels
can be visualized *in vivo*. Twenty minutes after acridine orange injection, static
leukocytes in the capillary bed can be observed as fluorescent dots. These labeled
cells are leukocytes because blocking CD18, expressed on leukocytes but not on
endothelial cells, causes them to disappear (see below).
15   Leukocyte dynamics in the retina were observed after CD18 F(ab')₂ blockade
as shown in the representative photos of Figure 9. Figure 9 shows retinal leukostasis
following CD18 blockade. Representative photos from acridine orange leukocyte
fluorography revealed static fluorescent leukocytes in the retinas of control and
diabetic rats. The leukostasis in non-diabetic rat retina (Figure 9A), was increased in
20   diabetic rat retina (Figure 9B), and unchanged following treatment with the control
F(ab')₂ (Figure 9C), however retinal leukostasis was reduced in diabetic rats treated
with anti-CD18 F(ab')₂ fragments (Figure 9D). As expected, retinal leukostasis was
increased in the diabetic vs. non-diabetic rat retinae (Figure 9B vs. 9A). Treatment
of the diabetic rats with the isotype control F(ab')₂ fragments did not lead to
25   detectable changes in the degree of leukostasis (Figure 9C vs. 9B). However,
treatment with the anti-CD18 F(ab')₂ fragments led to a striking decrease in retinal
leukostasis (Figure 9D vs. 9C). Measurements of leukostasis were obtained
throughout the entire retinae to avoid any potential sampling error and the means
and standard deviations from independent experiments were compared (Figure 10).
30   Figure 10 shows the quantitation of retinal leukostasis following CD18 blockade.

-46-

When CD18 bioactivity was inhibited via systemic administration of 5 mg/kg of the
anti-CD18 neutralizing F(ab')$_2$ (clone WT.3), retinal leukostasis was inhibited in
diabetic rat retinas. This confirmed that anti-CD18 blockade significantly decreased
leukostasis in diabetic rats by 62% (n=5, p=0.001 vs. animals receiving control

5   F(ab')$_2$) (Figure 10). The body weight, plasma glucose level, blood pressure, and
leukocyte counts for the control and diabetic animals are shown in Table 3. The
diabetic animals all had significantly elevated blood glucose levels and decreased
body weight as compared with the normal rats, as is the norm. Blood pressure was
similar among groups. The peripheral leukocyte counts in the diabetic anti-CD18

10 F(ab')2-treated animals were increased compared to the untreated diabetic animals, a
result consistent with successful CD18 blockade.

–47–

Table 3. Characteristics of control, diabetic, control mAb treated diabetic, and anti-CD18 F(ab')$_2$-treated diabetic rats

	Control	Diabetes	p-value	n
CD11a	115.0±12.8	185.9±18.5	0.006	5
CD11b	182.6±39.2	281.9±84.9	0.045	5
CD18	193.2±34.2	267.1±34.3	0.009	5

Values are means ± SD of mean channel fluorescence.

–48–

The results of the blocking adhesion studies indicate that Mac-1 is the predominant CD18 integrin involved in diabetic neutrophil adhesion to activated RPEC monolayers. At present, the reason for a lack of a CD11a-dependent component in diabetic vs. non-diabetic neutrophil adhesion is not known. The

5   residual non-CD18-dependent neutrophil adhesion may be due to the VLA_4-VCAM adhesion pathway because rat neutrophils constitutively express VLA_4 on their surface.

Conclusion: Neutrophils from diabetic animals exhibit higher levels of surface integrin expression and integrin-mediated adhesion. *In vivo*, CD18 blockade

10   significantly decreases leukostasis in the diabetic retinal microvasculature. Integrin adhesion molecules serve as therapeutic targets for the treatment and/or prevention of early diabetic retinopathy.

Example 3: Vascular Endothelial Growth Factor (VEGF)-induced retinal vascular permeability is mediated by ICAM-1

15   SUMMARY:

Two prominent VEGF-induced retinal effects are vascular permeability and capillary non-perfusion. The mechanisms by which these effects occur are not completely known. Using a rat model, it is shown that intravitreous injections of VEGF precipitate an extensive retinal leukocyte stasis (leukostasis) that coincides

20   with enhanced vascular permeability and capillary non-perfusion. The leukostasis is accompanied by the upregulation of intercellular adhesion molecule-1 (ICAM-1) expression in the retina. The inhibition of ICAM-1 bioactivity with a neutralizing antibody prevents the permeability and leukostasis increases by 79% and 54%, respectively. These data are the first to demonstrate that a non-endothelial cell type

25   contributes to VEGF-induced vascular permeability. Additionally, they identify a potential mechanism for VEGF-induced retinal capillary non-perfusion.

In experimental diabetes, the increased presence of static leukocytes in the retinal circulation is correlated with increased vascular permeability. The leukostasis and vascular permeability changes coincide with the upregulation of retinal ICAM-

1. When ICAM-1 bioactivity is blocked with an antibody, retinal leukostasis and vascular permeability are reduced by 49% and 86%, respectively.

When the retina is bathed in pathophysiologic concentrations of vascular endothelial growth factor (VEGF), enhanced vascular permeability and capillary

5   non-perfusion are among the vascular changes induced. The mechanisms by which these changes occur are largely unknown. The current studies examined the mechanisms underlying VEGF-induced retinal permeability and non-perfusion. Given the ability of VEGF to increase ICAM-1 expression in the retinal vasculature, the role of ICAM-1 in VEGF-induced vascular permeability and non-perfusion was

10   examined *in vivo*.

METHODS:

Animals. Long-Evans rats weighing approximately 200 g were used for these experiments. They were allowed free access to food and water in an air-conditioned room with a 12-hour light/12-hour dark cycle until they were used for

15   the experiments.

Intravitreous Injection Procedure. The rats were anesthetized with xylazine hydrochloride (4 mg/kg) (Phoenix Pharmaceutical, St. Joseph, MO) and ketamine hydrochloride (25 mg/kg) (Parke-Davis, Morris Plains, NJ). Intravitreous injections were performed by inserting a 30-gauge needle into the vitreous at a site 1 mm

20   posterior to the limbus of the eye. Insertion and infusion were performed and directly viewed through an operating microscope. Care was taken not to injure the lens or the retina. The head of the needle was positioned over the optic disc, and a 5 μl volume was slowly injected into the vitreous. Any eyes that exhibited damage to the lens or retina were discarded and not used for the analyses.

25   Acridine Orange Leukocyte Fluorography (AOLF) and Fluorescein Angiography. Leukocyte dynamics were evaluated using acridine orange leukocyte fluorography (AOLF). Nishiwaki H, *et al., Invest Ophthalmol Vis Sci*, 37:1341-1347 (1996); Miyamoto K, *et al., Invest Opthalmol Vis Sci* 39:2190-2194 (1998). Intravenous injection of acridine orange causes leukocytes and endothelial cells to

30   fluoresce through the non-covalent binding of the molecule to double stranded

−50−

nucleic acid. When a scanning laser ophthalmoscope is utilized, retinal leukocytes and blood vessels can be visualized *in vivo*. Twenty minutes following acridine orange injection, static leukocytes in the capillary bed are observed.

5   Twenty-four hours before leukocyte dynamics were observed, a heparin-lock catheter was surgically implanted in the right jugular vein for the administration of acridine orange and sodium fluorescein dye. The catheter was subcutaneously externalized to the back of the neck. The rats were anesthetized for this procedure with xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (25 mg/kg).

       Immediately before AOLF, each rat was again anesthetized, and the pupil of

10   the left eye was dilated with 1% tropicamide (Alcon, Humancao, Puerto Rico) to observe leukocyte dynamics. A focused image of the peripapillary fundus of the left eye was obtained with a scanning laser ophthalmoscope (SLO; Rodenstock Instrument, Munich, Germany). Acridine orange (Sigma, St. Louis, MO) was dissolved in sterile saline (1.0 mg/ml) and 3 mg/kg was injected through the jugular

15   vein catheter at a rate of 1 ml/min. The fundus was observed with the SLO using the argon blue laser as the illumination source and the standard fluorescein angiography filter in the 40° field setting for 1 minute. Twenty minutes later, the fundus was again observed to evaluate retinal leukostasis. The images were recorded on videotape at the rate of 30 frames/sec. The recordings were analyzed on a computer

20   equipped with a video digitizer (Radius, San Jose, CA) that digitizes video images in real time (30 frames/sec) at 640 x 480 pixels with an intensity resolution of 256 steps. For evaluating retinal leukostasis, an observation area around the optic disc measuring five disc diameters in radius was outlined by drawing a polygon bordered by the adjacent major retinal vessels. The area was measured in pixels and the

25   density of trapped leukocytes was calculated by dividing the number of static leukocytes, which were recognized as fluorescent dots, by the area of the observation region. The density of leukocytes was calculated in eight peripapillary observation areas and an average density was obtained by averaging the eight density values.

30   Immediately after observing and recording the static leukocytes, fluorescein angiography was performed to study the relationship between static leukocytes and

−51−

the retinal vasculature. Twenty μl of 1% sodium fluorescein was injected into the
jugular vein catheter and the images were captured using the SLO as described
above.

Quantitation of Retinal ICAM-1 mRNA Levels. Retinas were gently
5   dissected free and cut at the optic disc immediately after enucleation and frozen in
liquid nitrogen. Total RNA was isolated from rat retinas according to the acid
guanidinium thiocyanate-phenol-chloroform extraction method. A 425-base pair
EcoRI/BamHI fragment of rat ICAM-1 cDNA was prepared by reverse
transcription-polymerase chain reaction. The PCR product was cloned into
10   pBluescript II KS vector. After linearization by digestion with EcoNI, transcription
was performed with T7 RNA polymerase in the presence of $[^{32}P]dUTP$ generating a
225-base pair riboprobe. An automated DNA sequencer verified the sequence of the
cloned cDNA. Ten micrograms of total cellular RNA was used for the ribonuclease
protection assay. All samples were simultaneously hybridized with an 18S riboprobe
15   (Ambion, Austin, TX) to normalize for variations in loading and recovery of RNA.
Protected fragments were separated on a gel of 5% acrylamide, 8M urea, 1x Tris-
borate-EDTA, and quantified with a PhosphorImager (Molecular Dynamics,
Sunnyvale, CA).

Quantitation of Retinal Vascular Permeability. Vascular leakage was
20   quantified using the isotope dilution technique. Tilton RG, et al., J Clin Invest
99:2192-2202 (1997). Briefly, purified monomer bovine serum albumin (BSA;
Sigma, St. Louis, MO) (1 mg) was iodinated with 1 mCi of ^{131}I or ^{125}I using the
iodogen method. Polyethylene tubing (0.58 mm internal diameter) was used to
cannulate the right jugular vein and the left or right iliac artery. The tubing was
25   filled with heparinized saline (400 U heparin/ml). The right jugular vein cannula
was used for tracer injection. The iliac artery cannula was connected to a one ml
syringe attached to a Harvard Bioscience model PHD 2000 constant withdrawal
pump preset to withdraw at a constant rate of 0.055 ml/min. At time 0, $[^{125}I]$ albumin
(50 million cpm in 0.3 ml saline) was injected into the jugular vein and the
30   withdrawal pump started. At the eight minute mark, 0.2 ml (50 million cpm in 0.3
ml saline) of $[^{131}I]BSA$ was injected into the jugular vein. At the ten-minute mark,

–52–

the heart was excised, the withdrawal pump was stopped, and the retina was quickly

dissected and sampled for γ-spectrometry. Tissue and arterial samples were weighed

and counted in a γ-spectrometer (Beckman 5500, Irvine, CA). The data were

corrected for background and a quantitative index of [^{125}I] tissue clearance was

5   calculated as previously described and expressed as μg plasma x g tissue wet weight

$^{-1}$ x min^{-1}. Briefly, [^{125}I] BSA tissue activity was corrected for [^{125}I] BSA contained

within the tissue vasculature by multiplying [^{125}I]BSA activity in the tissue by the

ratio of [^{125}I]BSA/[^{131}I]BSA in an arterial plasma sample. The vascular-corrected

[^{125}I]BSA activity was divided by the time-averaged [^{125}I]BSA plasma activity

10 (obtained from a well-mixed sample of plasma taken from the withdrawal syringe)

and by the tracer circulation time (10 min) and then normalized per gram tissue wet

weight.

Anti-ICAM-1 Antibody Inhibition of Retinal Vascular Permeability and

Leukostasis. To study the *in vivo* effect of ICAM-1 blockade on VEGF-induced

15 retinal vascular permeability and leukostasis, a well characterized rat ICAM-1

neutralizing monoclonal antibody (mAb) was used utilized (1A29; R&D Systems,

Minneapolis, MN). Tamatani T, *et al., Int Immunol,* 165-171 (1990); Kawasaki K,

et al., J Immunol, 150:1074-1083 (1993); Kelly KJ, *et al., Proc Natl Acad Sci USA,*

91:812-816 (1994). The animals were randomly divided into five groups. The first

20 group received no treatment. The second group received 5 μl of phosphate-buffered

saline (PBS) injected into the vitreous of the left eye. The third group received 50 ng

VEGF$_{165}$ in 5 μl PBS injected into the vitreous of the left eye (12.5 nM final

concentration). The fourth group received 50 ng VEGF in PBS injected into the

vitreous of the left eye plus 5 mg/kg isotype-matched normal mouse IgG1(R&D

25 Systems) given intravenously. The fifth group received 50 ng VEGF in PBS injected

into the vitreous of the left eye plus 5 mg/kg of the anti-ICAM-1 mAb given

intravenously. Twenty-four hours later, retinal leukocyte dynamics and vascular

permeability were quantified.

Statistical Analysis. All results are expressed as the mean ± SD. Unpaired

30 groups of two were compared using the two sample t-test or the two sample t-test

with Welch's correction. To compare three or more groups, analysis of variance

–53–

(ANOVA) followed by the post hoc test with Fisher's protected least significant difference (PLSD) procedure was used. Differences were considered statistically significant when P values were less than 0.05.

RESULTS:

5   VEGF-induced Retinal Leukostasis. Figure 11A shows AOLF appearance of a normal retinal prior to injection of 50 ng VEGF. Figure 11B shows AOLF appearance of the same retinal area 48 h following intravitreous VEGF injection. Numerous static leukocytes are visible, as well as vessel dilation and tortuosity. A single 50 ng intravitreous injection of $VEGF_{165}$ (R& D Systems, Minneapolis, MN)

10   in 5 μl PBS was able to induce marked retinal leukostasis 48 h later (Figure 11). Vessel dilation and tortuosity were also evident. A dose-response study demonstrated that a 2.6-fold increase in leukostasis could be induced with as little as 10 ng VEGF (2.5 nM) (Figure 12, n=5, p<0.05). A plateau was reached with 50-100 ng VEGF (~4 - 5-fold, n=5, p=<0.001 to 0.0001). Based on these data, the 50 ng

15   dose was chosen for the time course experiments. Intravitreous injections of 50 ng VEGF were followed by AOLF 6, 24, 48, 72, and 120 h later. Twenty-four hours following intravitreous injection, VEGF increased retinal leukostasis 4.8-fold (Figure 13, n=5, p<0.01 vs. vehicle control). The VEGF-induced leukostasis increases peaked 48 h post-injection and persisted for at least 120 h (n=5, p<0.01).

20   To confirm that this effect was due to VEGF alone, four rats received a mixture of VEGF with a 50:1 molar excess of a previously characterized VEGF neutralizing monoclonal antibody (A4.6.1, Genentech, South San Francisco, CA) (Figure 14). Co-injection of the anti-VEGF antibody completely abrogated the VEGF-induced leukostasis 48 h later (n=4, p<0.001).

25   VEGF-induced Retinal Capillary Perfusion. Figure 15 shows leukocyte-induced capillary non-perfusion. Figure 15A shows the retina 48 hours after fifty ng VEGF was delivered via intravitreous injection as measured with AOLF. AOLF was immediately followed by fluorescein angiography and Figure 15B shows areas of capillary non-perfusion downstream from static leukocytes. Fluorescein

30   angiography performed 20 minutes following AOLF revealed relatively large areas

−54−

of downstream capillary non-perfusion associated with some of the static leukocytes (Figure 15). The majority of the leukocytes observed appeared to be in the intravascular space. Normal and vehicle injected eyes did not exhibit non-perfusion.

VEGF-induced Retinal ICAM-1 Gene Expression. Twenty hours following intravitreous injection of 50 ng VEGF or PBS vehicle alone, total RNA was isolated from each rat retina and ICAM-1 gene expression was quantitated using the ribonuclease protection assay (Figure 16A). When normalized to 18S, retinal ICAM-1 levels in the VEGF-injected eyes were 2.8-fold greater than in the eyes injected with vehicle alone (Figure16B, n=5, p<0.02).

ICAM-1 Blockade of VEGF-induced Vascular Permeability and Leukostasis. Animals receiving intravitreous VEGF had a 3.2-fold increase in vascular permeability 24 h following injection (Figure 17A. n=4, p<0.0001 vs. vehicle control). Similarly, there was a 4.3-fold increase in retinal leukostasis (Figure 17B, n=5, p<0.0001 vs. vehicle control). Intravenous treatment with the non-immune control antibody did not significantly alter the degree of VEGF-induced permeability (Figure 17A, n=3, p>0.05) or leukostasis (Figure 17B, n=4, p>0.05). However, the animals receiving intravenous anti-ICAM mAb had a 79% reduction in VEGF-induced retinal vascular permeability (Figure 17A, n=4, p<0.0001 vs. untreated) and a 54% reduction in VEGF-induced retinal leukostasis (Figure 17B, n=4, p<0.01 vs. untreated).

Example 4: CD18 and ICAM-1 dependent corneal neovascularization and inflammation following limbal injury

Materials and Methods.

Corneal Neovascularization Model

Male CD 18-deficient and ICAM-1-deficient mice were used (Jackson Labs, Bar Harbor, ME) and strain-specific normal male C57BL/6 mice served as controls. The mice were anesthetized with 50 mg/kg intraperitoneal pentobarbital sodium and a drop of proparicaine was instilled into the left eye. A number 15 Bard-Parker blade (vendor and city) was used to debride the corneal epithelium. Two microliters of

–55–

0.15 M NaOH was then applied topically and the limbal epithelium was removed
with a Tooke Corneal Knife, 2.5 x 15 mm Dissecting Blade (Arista, NY) A rotary
motion parallel to limbus was utilized. Erythromycin ophthalmic ointment was
instilled postoperatively.

5   Measurement of Corneal Neovascularization

            For measurement of neovascularization, mice were injected approximately 8
       μg of the endothelial cell-specific marker BS-1 lectin conjugated to FITC (Vector
       Laboratory) per 1g of body weight on day 7 after scraping or day 2 after
       implantation of VEGF. In 30 minutes after the injection of the dye, the eyes were
10   harvested and fixed with 10 % neutral buffered formalin, and then the cornea was
       flat-mounted on slide glasses. Fluorescence in the flat-mounted cornea was captured
       using CCD camera attached to a Leica Fluorescence microscope and saved to
       Macintosh 6500 (Apple computer) as a .tif image file. The images were taken with
       the same settings including exposure time on both study group and control one. The
15   digital images were processed using OpenLab Software and integrated optical
       density in the images was measured.

       Peripheral Leukocyte Counts

            Peripheral blood samples were collected from tail vessels into Eppendorf
       tube with EDTA when cornea that was served for confirming infiltration of PMN
20   was enucleated. For total leukocyte count blood was incubated with Turk solution
       and then counted manually using Hemocytemeter. The preparation of a thin, air-
       dried edge smear was made to perform the microscopic manual differential and
       stained with Giemsa solution. PMN count was then calculated from the differential.

       Corneal Leukocyte Counts

25   To determine the counts of PMN infiltration in cornea the eyes were
       enucleated on day 2 after scraping or implantation of VEGF, and stored in 10 %
       neutral buffered formalin. The tissue was embedded in paraffin, and 5-μm-thick
       sections were cut and then transferred to slide glasses. The tissue sections were

–56–

stained with Giemsa stain. The slides were then observed microscopically, and the number of PMNs was counted in 5 fields (2 of periphery, 2 of midperiphery and 1 of center) in the cornea from inflammatory models and in 1 field between VEGF pellet and corneal limbus in the cornea from VEGF-induced Neovascularization models.

5   Statistical Analysis

Student t-test and ANOVA were used for the comparison. Probability less than 0.05 was considered significant.

RESULTS:

Corneal Neovascularization in CD18 KO, ICAM-1 KO and Normal Mice

10   To determine if CD18 and ICAM-1 were important in the development of the corneal neovascularization associated with limbal injury, limbal injury was followed by quantitation of corneal neovascularization 7 d later. Compared to the strain-specific controls, the CD18 null mice 39% fewer vessels (n=5, p=0.0054). Similarly, the ICAM-1 null mice had 33% less neovascularization that the control

15   mice (n=5, p=0.013).

Corneal PMN Density in CD18 KO, ICAM-1 KO and Normal Mice

To determine if the inhibition of corneal neovascularization was associated with the decreased transmigration of PMN into the cornea, corneal PMN counts were performed 2 d following limbal injury. This time point was chosen because it

20   manifested maximum corneal opacity and corneal leukocyte infiltration. Compared to the strain-specific controls, the CD null mice had 66% fewer PMN (n=5, p=0.0016). The ICAM-1 null mice had 65% fewer PMN (n=5, p=0.0019) compared to the strain-specific controls.

25   Peripheral Blood PMN Counts in CD18 KO, ICAM-1 KO and Normal Mice

To determine if peripheral PMN cell counts were altered in the animals, standard PMN counts were calculated from the differential. The average count in the

–57–

C57BL6/J controls was 6263 ± 2313.18 vs. counts of 9315 ±1486 and 10,794 ±2199 in the CD18 and ICAM knockout mice, respectively.

DISCUSSION:

5   The data indicate that CD18 and ICAM-1 amplify the corneal neovascularization that occurs following limbal injury. The process is associated with higher corneal leukocyte counts, and the latter are likely causal, in part, for the increased neovascularization. The data also indicate that the CD18 and ICAM-1 KO mice have a higher proportion of circulating leukocytes, a result consistent with

10   absence of CD18 and ICAM-1 systemically. It also confirms that the corneal leukocytes likely transmigrated, and are not the result of systemic leukocytopenia. Taken together, these data identify CD18 and ICAM-1 mediators of the inflammatory corneal neovascularization in a clinically relevant model of limbal injury.

15   Experiments described herein show that limbal injury upregulates VEGF. When VEGF is inhibited, corneal neovascularization is reduced. VEGF is known to act directly on the endothelial cells and the vasculature, resulting in neovascularization. However, leukocytes augment this process. The mechanism involves VEGF. Leukocytes, via their own VEGF, serve to amplify the direct effects

20   of non-leukocyte VEGF on the vasculature. VEGF has been demonstrated in neutrophils, monocytes, eosinophils, lymphocytes and platelets. It has also been identified in the neutrophils and monocytes that infiltrate the cornea following limbal injury. The fact that some leukocytes possess high affinity VEGF receptors and migrate in response to VEGF is consistent with this scenario. Endogenous

25   VEGF triggers leukocyte adhesion, transmigration and further VEGF release, producing a positive feedback loop.

EQUIVALENTS

        While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled

30   in the art that various changes in form and details may be made therein without

departing from the spirit and scope of the invention as defined by the appended claims.

The relevant teachings of all the references, patents and/or patent applications cited herein are incorporated herein by reference in their entirety.

–59–

CLAIMS

What is claimed is:

1.   A method of inhibiting leukocyte interaction, comprising contacting a
      leukocyte, an endothelial cell or a leukocyte adhesion-inducing cytokine with
5   a compound, wherein the compound is selected from the group consisting of:
      an integrin antagonist, a selectin antagonist, an adhesion molecule
      antagonist, leukocyte adhesion-inducing cytokine antagonist and growth
      factor antagonist

2.   The method of Claim 1, wherein the leukocyte adhesion-inducing cytokine is
10   TNF-α , IL-1β or MCP-1.

3.   A method of inhibiting leukocyte interaction, comprising contacting an
      endothelial cell with an adhesion molecule antagonist.

4.   The method of Claim 3, wherein the adhesion molecule antagonist is specific
      to ICAM-1.

15 5. A method of inhibiting leukocyte interaction, comprising contacting an
      endothelial cell with an integrin subunit antagonist.

6.   The method of Claim 5, wherein the integrin subunit antagonist is specific to
      CD18 and/or CD11b.

7.   A method of inhibiting leukocyte interaction, comprising contacting a
20   leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist.

–60–

8.   The method of Claim 7, wherein the leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist is selected from a group consisting of: TNF-1α, IL-1β, MCP-1 and VEGF antagonist.

9.   Use of an integrin antagonist, a selectin antagonist, an adhesion molecule antagonist or a leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist, for the manufacture of a medicament, for inhibiting leukocyte interaction.

10.   The use of Claim 9, wherein the integrin antagonist is a LFA-1 antagonist, a Mac-1 antagonist or a p150,95 antagonist.

11.   The use of Claim 9, wherein the integrin antagonist comprises an integrin subunit antagonist.

12.   The use of Claim 11, wherein the integrin subunit antagonist comprises a CD18 antagonist or a CD11b antagonist.

13.   The use of Claim 9, wherein the selectin antagonist is a P-selectin antagonist, an E-selectin antagonist or a L-selectin antagonist.

14.   The use of Claim 9, wherein the adhesion molecule antagonist is an ICAM-1 antagonist, an ICAM-2 antagonist, an ICAM-3 antagonist, a PCAM antagonist or a VCAM antagonist.

15.   The use of Claim 9, wherein the leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist is a TNF-1α antagonist, IL-1β antagonist, MCP-1 antagonist or VEGF antagonist.

16.   Use of a compound, for the manufacture of a medicament, for reducing or preventing retinal injury, wherein the injury involves retinal edema or retinal

ischemia, comprising a compound that inhibits the binding of a leukocyte to
an endothelial cell or to another leukocyte.

17.   The use of Claim 16, wherein the compound is an integrin antagonist, a
        selectin antagonist, an adhesion molecule antagonist or a leukocyte
        adhesion-inducing cytokine antagonist or growth factor antagonist.

18.   The use of Claim 17, wherein the integrin antagonist is a LFA-1 antagonist,
        a Mac-1 antagonist or a p150,95 antagonist.

19.   The use of Claim 17, wherein the integrin antagonist comprises an integrin
        subunit antagonist.

20.   The use of Claim 19, wherein the integrin subunit antagonist comprises a
        CD18 antagonist or a CD11b antagonist.

21.   The use of Claim 17, wherein the selectin antagonist is a P-selectin
        antagonist, an E-selectin antagonist or a L-selectin antagonist.

22.   The use of Claim 17, wherein the adhesion molecule antagonist is an ICAM-
        1 antagonist, an ICAM-2 antagonist, an ICAM-3 antagonist, a PCAM
        antagonist or a VCAM antagonist.

23.   The use of Claim 17, wherein the leukocyte adhesion-inducing cytokine
        antagonist or growth factor antagonist is a TNF-1α antagonist, IL-1β
        antagonist, MCP-1 antagonist or VEGF antagonist.

24.   Use of a compound, for use in therapy, e.g., vein occlusions, sickle cell
        retinopathy, radiation retinopathy, diabetic retinopathy, VEGF-induced
        diseases and retinopathy prematurity, comprising a compound that inhibits
        the binding of a leukocyte to an endothelial cell or to another leukocyte.

−62−

25. The use of Claim 24, wherein the compound is an integrin antagonist, a selectin antagonist, an adhesion molecule antagonist or a leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist.

26. Use of a CD18 antagonist and an ICAM-1 antagonist, or a CD18 antagonist, for the manufacture of a medicament, for reducing neovascularization.

27. The use of Claim 26, wherein the CD18 antagonist or the ICAM-1 antagonist is an antibody, an antibody fragment, a peptide mimetic molecule, an antisense molecule, a ribozyme, an aptamer and/or a small molecule antagonist.

28. The use of Claim 27, wherein the neovascularization is reduced in the cornea, the retina or the choroid.

29. Use of compound in therapy e.g., age-related macular degeneration, choroidal neovascularization, sickle cell retinopathy, retina vein occlusion, diabetic retinopathy, a condition associated with limbal injury, a condition associated with increased neovascularization, traumatic alkali injury, Stevens Johnson syndrome or ocular cicatricial pemphagoid, comprising a CD18 antagonist and an ICAM-1 antagonist, or a CD18 antagonist.

30. The use of Claim 29, wherein the CD18 antagonist or the ICAM-1 antagonist is an antibody, an antibody fragment, a peptide mimetic molecule, an antisense molecule, a ribozyme, an aptamer and/or a small molecule antagonist.

31. The use of Claim 29, wherein the neovascularization is reduced in the cornea, the retina or the choroid.

—63—

32. A method for reducing or preventing retinal injury in a mammal, wherein the injury involves retinal edema or retinal ischemia, comprising administering to the mammal a compound that inhibits the binding of a leukocyte to an endothelial cell or to another leukocyte, wherein a reduction in retinal edema or retinal ischemia occurs.

33. The method of Claim 32, wherein the compound is an integrin antagonist, a selectin antagonist, an adhesion molecule antagonist or a leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist.

34. The method of Claim 33, wherein the integrin antagonist is selected from a group consisting of: LFA-1 antagonist, Mac-1 antagonist and p150,95 antagonist.

35. The method of Claim 34, wherein the integrin antagonist comprises an integrin subunit antagonist.

36. The method of Claim 35, wherein the integrin subunit antagonist comprises a CD18 antagonist or a CD11b antagonist.

37. The method of Claim 33, wherein the selectin antagonist is selected from a group consisting of: P-selectin antagonist, E-selectin antagonist and L-selectin antagonist.

38. The method of Claim 33, wherein the adhesion molecule antagonist is selected from the group consisting of: ICAM-1 antagonist, ICAM-2 antagonist, ICAM-3 antagonist, PCAM antagonist, and VCAM antagonist.

39. The method of Claim 33, wherein the leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist is selected from a group consisting of: TNF-1α, IL-1β, MCP-1 and VEGF antagonist.

-64-

40. A method for reducing or preventing retinal injury in a mammal, comprising administering to the mammal an adhesion molecule antagonist and/or an integrin antagonist, wherein the adhesion molecule antagonist and/or the integrin antagonist inhibits leukocyte interaction, thereby reducing or
5   preventing retinal injury.

41. The method of Claim 40, wherein the adhesion molecule antagonist is an ICAM-1 antagonist, ICAM-2 antagonist, ICAM-3 antagonist, PCAM antagonist and VCAM antagonist.

42. The method of Claim 40, wherein the antagonist is an antibody or antibody
10   fragment specific for ICAM-1.

43. The method of Claim 40, wherein the antagonist is an antisense molecule that hybridizes to the nucleic acid sequence that encodes ICAM-1, or a peptide mimetic molecule, ribozyme, an aptamer or a small molecule antagonist that inhibits ICAM-1.

15 44. The method of Claim 40, wherein the integrin antagonist is selected from a group consisting of: LFA-1 antagonist , Mac-1 antagonist and p150,95 antagonist.

45. The method of Claim 44, wherein the integrin antagonist comprises an integrin subunit antagonist.

20 46. The method of Claim 45, wherein the integrin subunit antagonist comprises a CD18 antagonist and/or a CD11b antagonist.

47. The method of Claim 46, wherein the antagonist is an antibody or antibody fragment specific for CD18 and/or CD11b

48. The method of Claim 46, wherein the antagonist is an antisense molecule that hybridizes to the nucleic acid sequence that encodes CD18 and/or CD11b, or a peptide mimetic molecule, a ribozyme, an aptamer or a small molecule antagonist that inhibits CD18 or CD11b.

49. The method of Claim 40, wherein the adhesion molecule antagonist and/or the integrin subunit antagonist is administered in a pharmaceutically acceptable carrier.

50. A method for preventing or treating an individual having retinal injury, wherein the injury is associated with retinal edema and/or retinal ischemia, comprising administering to the individual a compound that inhibits Mac-1 or a pathway thereof, wherein a reduction in retinal edema and/or retinal ischemia occurs.

51. The method of Claim 50, wherein the compound inhibits ICAM-1, CD18, CD11b, and/or VEGF.

52. The method of Claim 51, wherein a decrease of ischemia and/or edema occurs.

53. The method of Claim 52, wherein the decrease of ischemia and/or edema is between about 10% and about 90%.

54. The method of Claim 50, wherein the individual has diabetic retinopathy.

55. A method for treating an individual having diabetic retinopathy comprising administering to the individual a compound that inhibits Mac-1 or a pathway thereof, wherein leukocyte interaction is reduced.

56. The method of Claim 55, wherein the compound comprises an antibody, an antibody fragment, a peptide mimetic molecule, an antisense molecule, a ribozyme, an aptamer and/or a small molecule antagonist.

57. The method of Claim 56, wherein the compound inhibits ICAM-1, CD18, CD11b, and/or VEGF.

58. A method for treating an individual having diabetic retinopathy comprising administering to the individual an ICAM-1, CD18, CD11b and/or VEGF antagonist, wherein the ICAM-1, CD18, CD11b and/or VEGF antagonist inhibits leukocyte interaction.

59. The method of Claim 58, wherein the antagonist is an antibody or antibody fragment specific for ICAM-1, CD18, CD11b and/or VEGF.

60. The method of Claim 58, wherein the antagonist is an antisense molecule that hybridizes to the nucleic acid sequence that encodes ICAM-1, CD18, CD11b and/or VEGF.

61. The method of Claim 58, wherein a decrease of ischemia and/or edema occurs.

62. The method of Claim 61, wherein the decrease of ischemia and/or edema is between about 10% and about 90%.

63. A method for treating an individual with retinal edema comprising administering to the individual an ICAM-1, CD18, CD11b and/or VEGF antagonist, wherein a decrease in the retinal edema occurs.

64. The method of Claim 63, wherein the antagonist is an antibody or antibody fragment that is specific for ICAM-1, CD18, CD11b and/or VEGF.

–67–

65.   The method of Claim 63, wherein the antagonist is an antisense molecule that hybridizes to a nucleic acid sequence that encodes ICAM-1, CD18, CD11b and/or VEGF.

66.   The method of Claim 63, wherein the decrease of a edema is between about 10% and about 90%.

67.   A method for treating an individual with retinal ischemia comprising administering to the individual an ICAM-1, CD18, CD11b and/or VEGF antagonist, wherein a decrease in ischemia occurs.

68.   The method of Claim 67, wherein the antagonist is an antibody or antibody fragment specific for ICAM-1, CD18, CD11b and/or VEGF.

69.   The method of Claim 67, wherein the decrease of ischemia is between about 10% and about 90%.

70.   The method of Claim 67, wherein the antagonist is an antisense molecule that hybridizes to a nucleic acid sequence which encodes ICAM-1, CD18, CD11b and/or VEGF.

71.   The method of treating diabetic retinopathy in an individual, comprising administering to the individual an ICAM-1, a CD18, CD11b and/or VEGF antagonist and at least one additional antagonist that inhibits the binding of a leukocyte to an endothelial cell or to another leukocyte.

72.   The method of Claim 71, wherein the additional antagonist is an integrin antagonist, selectin antagonist, a leukocyte adhesion-inducing cytokine antagonist or growth factor antagonist, or another adhesion molecule antagonist.

–68–

73.   The method of Claim 72, wherein the integrin is selected from a group
       consisting of: LFA-1, Mac-1 and p150,95.

74.   The method of Claim 73, wherein the integrin antagonist comprises an
       integrin subunit antagonist.

75.   The method of Claim 74, wherein the integrin subunit antagonist comprises
       another CD18 antagonist or another CD11b antagonist.

76.   The method of Claim 72, wherein the selectin is selected from a group
       consisting of: P-selectin, E-selectin and L-selectin.

77.   The method of Claim 72, wherein the leukocyte adhesion-inducing cytokine
       antagonist or growth factor antagonist is selected from a group consisting of:
       TNF-1α, IL-1β, MCP-1 and another VEGF antagonist

78.   The method of Claim 72, wherein the adhesion molecule antagonist is a
       PCAM antagonist, VCAM antagonist, an ICAM-2 antagonist, an ICAM-3
       antagonist or another ICAM-1 antagonist.

79.   A method of preventing or reducing retinal leukostasis in a mammal
       comprising administering to the mammal an effective amount of an ICAM,
       CD18, CD11b and/or VEGF antagonist.

80.   The method of Claim 79, wherein the ICAM antagonist is an ICAM-1
       antagonist.

81.   The method of Claim 79, wherein the antagonist is an antibody or antibody
       fragment specific for ICAM-1, CD18, CD11b and/or VEGF.

82. The method of Claim 79, wherein the antagonist is an antisense molecule that hybridizes to a nucleic acid sequence which encodes ICAM-1, CD18, CD11b and/or VEGF.

83. The method of Claim 79, wherein retinal leukostasis is reduced by between about 10% and 90%.

84. A method of decreasing retinal leukocyte adhesion in a mammal, comprising administering to the mammal an effective amount of an antagonist that is specific for CD11b, CD18 or a combination thereof.

85. The method of Claim 84, wherein the antagonist is an antibody or antibody fragment.

86. The method of Claim 84, wherein retinal leukocyte adhesion is decreased between about 10% and 90%.

87. A method of treating or preventing neovascularization in a mammal, comprising administering to the mammal a CD18 antagonist and an ICAM-1 antagonist, wherein neovascularization is reduced.

88. The method of Claim 87, wherein the CD18 antagonist or the ICAM-1 antagonist is an antibody, an antibody fragment, a peptide mimetic molecule, an antisense molecule, a ribozyme, an aptamer and/or a small molecule antagonist.

89. The method of Claim 87, wherein the mammal has a disease, condition or disorder selected from the group consisting of: age-related macular degeneration, choroidal neovascularization, sickle cell retinopathy, retina vein occlusion, diabetic retinopathy, a condition associated with limbal

−70−

injury, a condition associated with increased neovascularization, traumatic alkali injury, Stevens Johnson syndrome and ocular cicatricial pemphagoid.

90. The method of Claim 87, wherein the neovascularization is reduced in the cornea, the retina or the choroid.

91. A method of treating or preventing neovascularization in a mammal, comprising administering to the mammal a CD18 antagonist, wherein neovascularization is reduced.

92. The method of Claim 91, wherein the CD18 antagonist is an antibody, an antibody fragment, a peptide mimetic molecule, an antisense molecule, a ribozyme, an aptamer and/or a small molecule antagonist.

93. The method of Claim 91, wherein the mammal has a disease, condition or disorder selected from the group consisting of: age-related macular degeneration, choroidal neovascularization, sickle cell retinopathy, retina vein occlusion, diabetic retinopathy, a condition associated with limbal injury, a condition associated with increased neovascularization, traumatic alkali injury, Stevens Johnson syndrome and ocular cicatricial pemphagoid.

94. The method of Claim 91, wherein the neovascularization is reduced in the cornea, the retina or the choroid.

FIG. IA
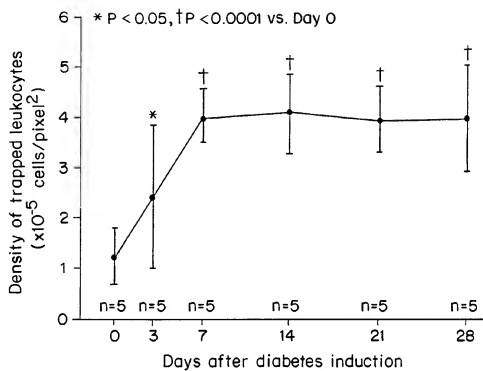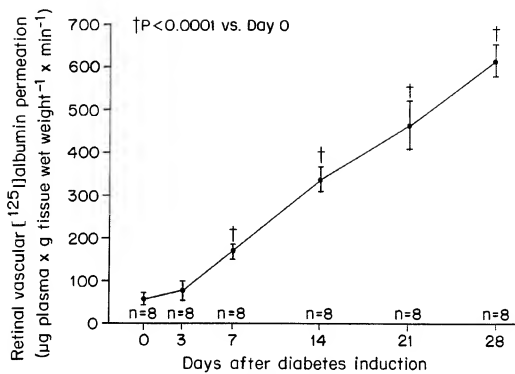
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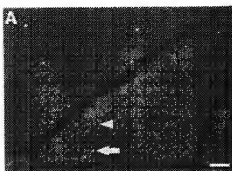
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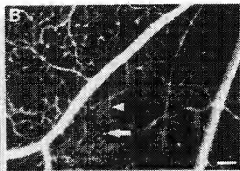
FIG. 2C   FIG. 2D

FIG. 3A
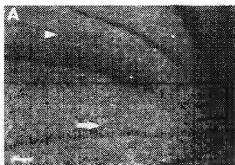
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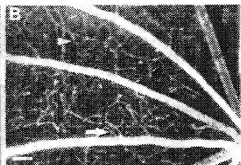
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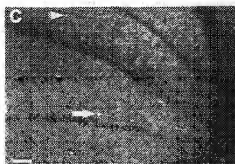
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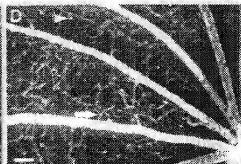
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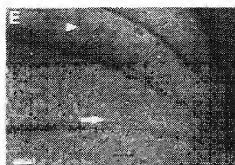
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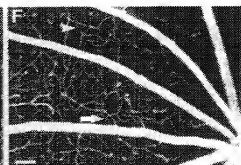
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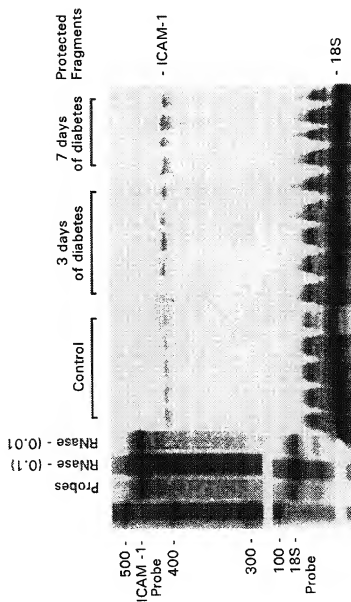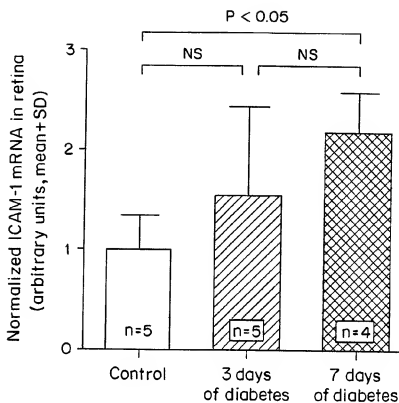
FIG. 4B

FIG. 5A   FIG. 5B
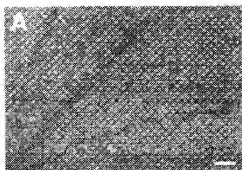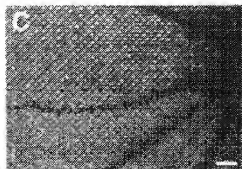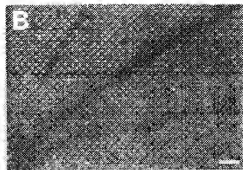
FIG. 5C   FIG. 5D
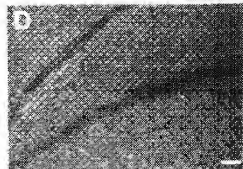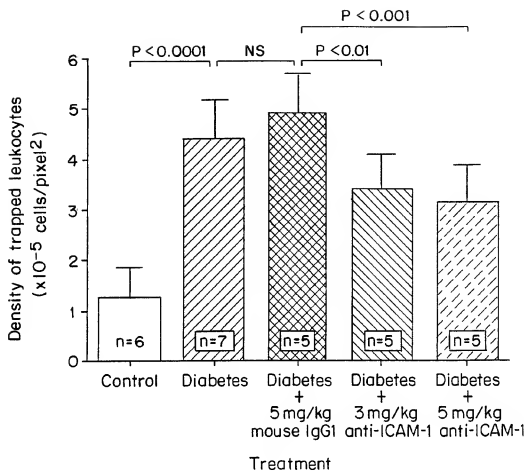
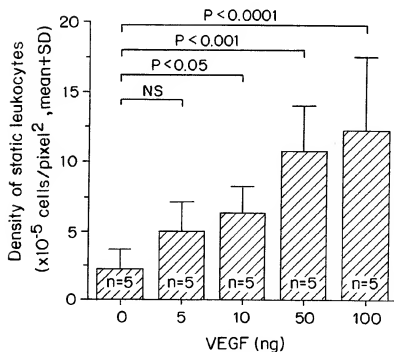FIG. 6A
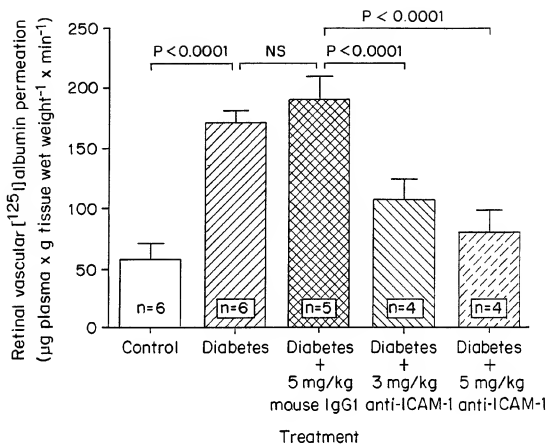
FIG. 6B
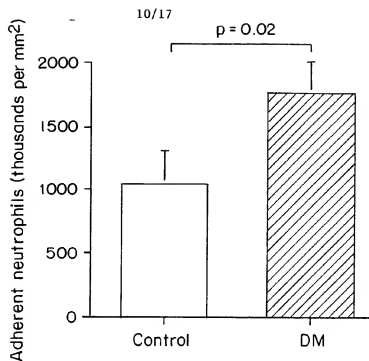
FIG. 7
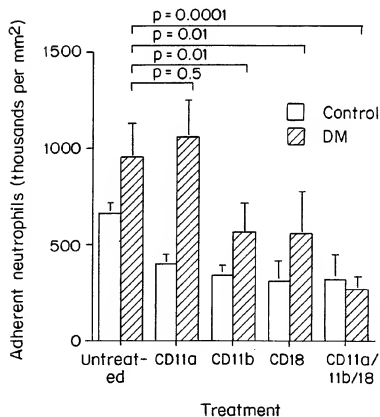
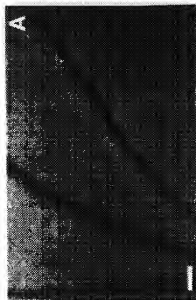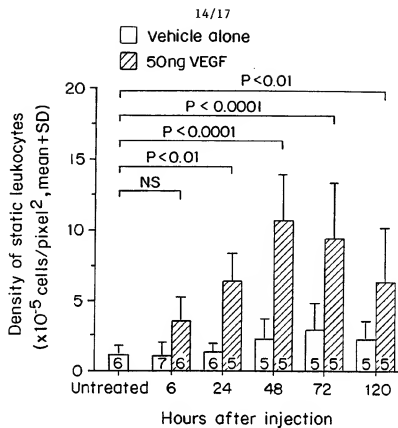
FIG. 8

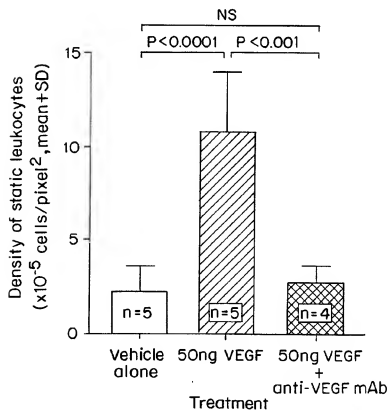FIG. 9B

FIG. 9A

FIG. 9D
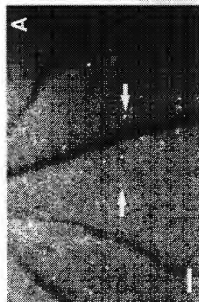
FIG. 9C
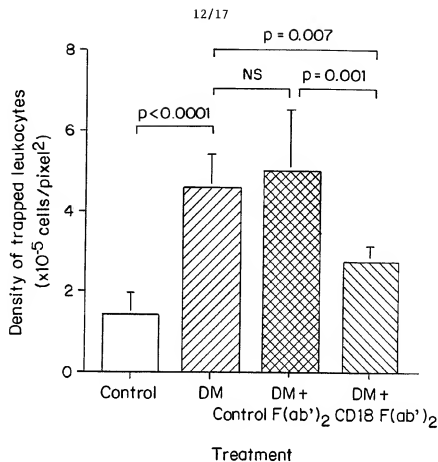
FIG. 10

FIG. 12

FIG. 11A

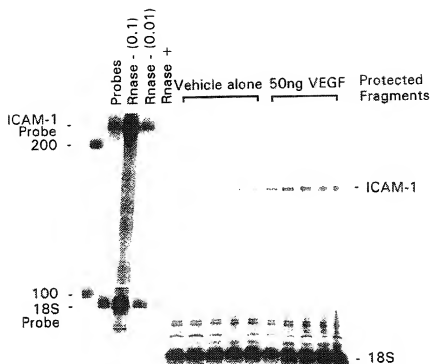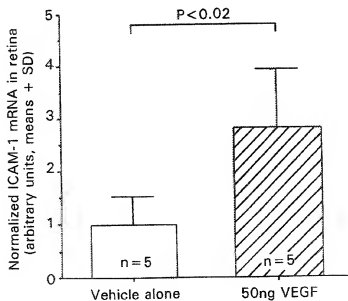FIG. 11B

FIG. 13

FIG. 14

FIG. 15B

FIG. 15A

FIG. 16A

FIG. 16B

FIG. 17A

FIG. 17B